**Arch. Inst. Razi, 1970, 22, 11-18**

# Recent Advances in Immunization of Horses Against African Horsesickness (*)

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Some progress has been made in the field of immunization of horses against African horsesickness (AHS) with killed vaccine since the First International Conference on Equine Infectious Diseases was held in Stresa in 1966. A brief up-to-date survey is, therefore, requisite but only some of the highlights in the preparation and application of killed vaccine will be dealt with in the present paper.

*Introduction*

The polyvalent live AHS vaccine prepared with 7 or 8 types of neurotropic virus in mouse brain [1] or in cell culture [13, 18] was the major achievement in the field of widespread prophylaxis of AHS. Without any intention of underestimating the importance of this achievement, we should emphasize that while this type of vaccine was the only means of liquidating epizootics in the Middle East (1959), North Africa (1965) and Spain (1966), it can not be considered to be an entirely satisfactory vaccine to be recommended for use in those areas with no previous history of AHS. The postvaccinal encephalitis [17, 20, 23] amongst the fully susceptible horses in the Middle East; blindness, emaciation, transient signs of untoward reactions [5] mortality among donkeys immunized with mouse brain vaccine [7], isolation of type 2 virus from the vaccinated equine [20], the extra virulence of some vaccine strains for highly susceptible animals, the possibility of presence of adventitious agents and brain tissues in the vaccine and finally breakdown in immunity induced by polyvalent vaccine [9], are some disadvantages attributed to the live vaccine. In Western countries, where the exotic virus has not been introduced, it is hard to ensure that by immunization of highly susceptible equines with live AHS vaccine will not circulate the virus among the equine population with the possibility of giving rise to eventual fresh outbreaks of the disease.

These facts support the rationale of an effort directed toward preparation of a suitable inactivated vaccine that might provide adequate protection to equines in endemic regions as well as be suitable for use as a prophylactic for protection of imported animals.

Killed vaccines for AHS were used even before live attenuated vaccine. WITWORTH [26], WALKER [25], KIND [10], DU TOIT and ALEXANDER [2], DU TOIT, ALEXANDER and NEITZ [3], have used emulsion of spleen of horses killed by the disease, inactivating the virus by the use of different concentrations of formalin. The

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(*) Proc. 2nd int. Conf. Equine Infectious Diseases, Paris 1969, pp. 212-221 (Karger, Basel/München/New York 1970).

same vaccine mixed with 1 in 5,000 of Saponin as adjuvant was used in Iran [21] and in Turkey [22]. In all cases the immunity was ineffective or transient in its duration. The inactivated virus was prepared from cell cultures and formalin killed. It induced resistance in horses to challenge doses of homologous virus but the serological responses of these horses were below the current level normally obsrved in long lasting immunity [19]. In the present study we have immunized horses with two doses of a monovalent killed vaccine mixed with adjuvant [15]. Some comments on the preparation and results will be presented here. More details about production and control of this killed vaccine have been given in another report [24].

*Procedure for Production of Killed Vaccine*

*Selection of Virus*

It is necessary to select the required virus types highly immunogenic. In this study the attenuated neurotropic strain (S 2) and the virulent viscerotropic strain (10/60) both type 9 were used. The specific response to monovalent vaccine is normally clear and uncomplicated. On the other hand, mixing several viruses in the vaccine will present complex problems in testing and control. Therefore, all virus components must be compatible with the others. To maintain the quality of vaccine all components must retain their potency in order to avoid readjusting and retesting of the vaccine during storage. Since the immunogenic potency of certain strains may be reduced or even lost at a very low passage level [5, 14], the passage in cell culture must be limited to a few. The immunogenic or pathogenic potency of cell culture adapted virus should be first evaluated in susceptible horses [5].

*Cell Culture*

Monkey kidney (MS Line) cells were grown in a medium consisting of Earle's solution with 0.5% lactalbumin hydrolysate, 0.5% Difco yeast extract and 10% inactivated calf serum.

In the maintenance medium, the calf serum was reduced to 1—2%. 100 units penicillin and 100 units streptomycin per ml were added.

*Preparation of Virus Suspension*

Roux bottles of MS cells were infected at a multiplicity of infection (m.o.i.) of 1. After 2 h. adsorption at 36° C, with occasional shaking, an appropriate amount of maintenance medium was added to each bottle. The bottles were reincubated at 36° C for 48 h. By this time more than 75% of the cell sheet showed specific cytopathic effect (CPE). The fluid was then removed and filtered on a clarifying pad (C 5) before inactivation. At this level the virus titer was $10^{6.5}$ to $10^{7.5}$ median tissue culture (TCID50) doses per ml.

*Inactivation of Virus*

In comparative tests the virus was inactivated in 12 days at + 4° C by the addition of formalin (HCHO, 37%) at a final concentration of 1:8,000 formaldehyde. Inactivation with the same concentration of formaldehyde occurs at 25° C in 38 h. During and after inactivation, samples of virus suspension were dialyzed overnight against

two changes of cold phosphate buffered saline (PBS) before being used for titration of residual virus. Ten tubes of MS cells were used for detecting residual virus. Although these cultures were negative after 7 to 10 days, subculturing the frozen and thawed cells of the first passage in MS cells revealed the occasional presence of residual virus in very small amounts.

The temperature of inactivation was, therefore, raised to 32° C. At this temperature the inactivation was complete in 48 h. Cultures and subcultures in MS cells or in mice by intracerebral inoculation were negative [24].

Inactivation of AHS virus by betapropiolactone has been discussed in a previous report [15]. Figures 1 and 2 represent residual infectivity of AHS virus suspension by formalin or BPL at 25° C.

*Adjuvant*

As we have mentioned, injection of horses with large amounts of killed AHS vaccine results in a low immunity. Large amounts of antigen (50 ml or more) may give

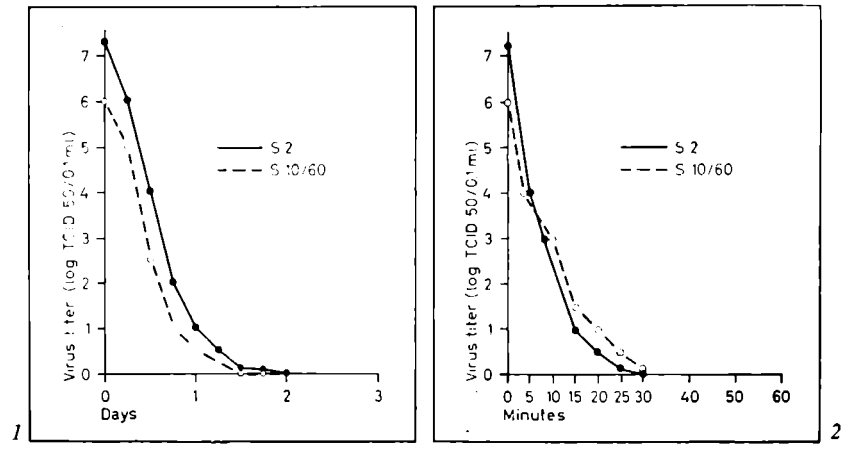


*Fig. 1.* Residual infectivity of AHS virus suspension during Formalin (1:3000) inactivation at 25° C, pH 7.2.

*Fig. 2.* Residual infectivity of AHS virus suspension during Betapropiolactone (2:1000) inactivation at 36° C, pH 7.4.

rise to untoward reactions. Successful immunization may also require several injections which are not practical to administer. To surmount these problems, we have turned to adjuvant in order to improve antibody production. The dose injected was reduced accordingly but it was essential to administer at least two injections at an interval of four weeks. Following a fundamental concept of immunology the first injection of any kind of antigen plays the role of 'inductor'. The interval between two injections is a 'determinant phase' and in this phase the type of cells playing a role in immune responses will be decided [5]. It is a few days to 4 weeks after the second injection of antigen that progressive rise in titer of specific antibody will appear. This response reaches a peak within a few weeks of the onset of immunity and then declines slowly. The antibody response will fall gradually below the level that currently available techniques can

measures. A third boosting will generally give rise to much higher antibody titer than the level recorded after the second response. This immunity will last longer and the decline in titer is normally slow. Among the many factors that can markedly influence both rise and duration of the immune response are adjuvants which are widely used in medical and veterinary prophylaxis. In this study we have used aluminum hydroxide, Al (OH)3.

*Immunization of Horses*

Horses were obtained from Khorassan, Northeast of Iran, where no previous outbreak of the disease had been recorded. The horses were 2–3 years old. The serum of all horses was screened for neutralizing antibody before vaccination as well as 4 weeks after each immunization. All horses were found to be free of antibodies for the virus type used in the tests.

Each horse was inoculated subcutaneously with 15 ml of monovalent vaccine. The temperature of all horses was recorded twice a day for 4 weeks. A second injection of 15 ml of the same vaccine was then given subcutaneously. A groupe of 50 horses that had been immunized previously with live cell culture attenuated virus vaccine were revaccinated with a dose of 15 ml of homologous killed vaccine mixed with aluminum hydroxide. *In vitro* serum neutralization tests were performed in MS cells as described previously [13].

*Results*

Post-Vaccinal and Post-Challenge Reactions

No fever or any abnormal reaction was noted during the 4-week period of post-vaccinal observation. Local swellings of various sizes, due to aluminum gel, were observed in 25% of the animals. These reactions faded 3 to 4 weeks later without any treatment. A few days after challenge with virulent virus, a rise of temperature not exceeding 40° C was recorded in some horses immunized with PBL-treated antigen; this fever was soon over and no other signs of illness were noticed. The control died, specific pathological changes were demonstrated in various organs and the virus was isolated from the blood.

*Immunological Response of the Horse to Inactivated AHS Vaccine*

*Response to a Single Dose of Vaccine*

A comparison was made between the immunogenic response induced in two groups of five horses previously vaccinated with formalin or BPL-treated vaccine. All horses were bled 4 weeks after immunization and tested for neutralizing antibodies. Two weeks later these horses and one control were challenged with virulent virus (S 10/60) by intravenous injection. The virulent virus was prepared from an 8% suspension of fresh suckling mouse brain, collected when the mice were *in extremis*. The suspension was centrifuged in the cold (2° C) for 15 min at 2500 rpm before inoculation. Each horse was challenged with 4 ml of virulent virus. Horses immunized with formalin-treated vaccine had the highest levels of neutralizing antibodies, but both groups resisted challenge with virulent virus. The control died with respiratory symptoms 17 days after inoculation of virus and the virus was recovered from its blood (table 1).

*Response to Two Doses of Vaccine*

Two groups of five horses were immunized, one group with two doses of 15 ml of formalin-inactivated, and another group with BPL-treated vaccine at an interval between injections of 4 weeks. The concentration of Al (OH)3 was the same in both preparations. Test bleeding was performed 4 weeks after each injection, 6 months after the last injection and just before challenge with virulent virus. The antibody response was higher to formalin-treated vaccine than to BPL-treated vaccine (table II). Six months after immunization the remaining antibodies in horses immunized with formalin-treated vaccine were of higher titer than in horses immunized with BPL-treated vaccine. However, both groups resisted challenge with homologous virus.

*Table I.* Immunity in horse immunized with a single dose of inactivated AHS vaccine

| Horse No. | Type of vaccine | Antibody titer 6 weeks after immunization | Reaction to challenge |
|---|---|---|---|
| 1 | | 128[1] | N |
| 2 | | 512 | N |
| 3 | Formalin-treated | 64 | N |
| 4 | | 64 | N |
| 5 | | 128 | N |
| 6 | | 4 | N |
| 7 | | 4 | N |
| 8 | BPL-treated | 16 | N |
| 9 | | 4 | N |
| 10 | | 16 | N |
| 11 | Control | — | Died |

1 Reciprocal of the dilution of serum which neutralizes 100 TCID 50.
N = Normal.

*Boosting Effect of Inactivated Vaccine in Horses Previously Immunized with Live Attenuated Vaccine*

Fifty horses and mules that had been immunized with cell culture adapted monovalent live vaccine (type 9-S2), 2 to 5 years before this study was conducted, were reinoculated with a dose of 15 ml of homologous inactivated killed vaccine. The vaccine used was a mixture of strains 10/60 and S2, inactivated separately at 25° C with the same concentration of Al (OH)3 as in previous experiments.

A four-fold or higher rise in neutralizing antibody titer was observed [16], 6 weeks after vaccine injection in majority of the equines under study.

*Discussion*

The immunization of horses with monovalent formalin or BPL-killed AHS vaccine can be done safely. The immune response of horses will be significantly increased if an adjuvant, such as aluminum hydroxyde, is added. Two injections, at an interval of 4 weeks, give a better immune response lasting more than six months and probably more

*Table II.* Immunity in horses immunized with two doses of inactivated AHS vaccine

| Horse No. | Type of vaccine | Antibody titer | | | Reaction to challenge 6 months after second immunization |
|---|---|---|---|---|---|
| | | 4 weeks after first immunization | 4 weeks after second immunization | 6 months after second immunization | |
| 1 | | 64[1] | 512 | 64 | N |
| 2 | | 32 | 64 | 8 | N |
| 3 | Formalin- | | | | |
| | treated | 32 | 128 | 32 | N |
| 4 | | 128 | 512 | 128 | N |
| 5 | | 64 | 256 | 32 | N |
| 6 | | 16 | 64 | 16 | N |
| 7 | | 4 | 16 | 4 | N |
| 8 | BPL-treated | 4 | 8 | 4> | N |
| 9 | | 4 | 8 | 4> | N |
| 10 | | 4 | 16 | 4> | N |
| 11 | Control | — | — | — | Died |

1 Reciprocal of the dilution of serum which neutralizes 100 TCID 50.
N = Normal.

than a year. There are, however, several problems to be dealth with before such a vaccine can be used on a large scale. The effects of combining several virus types after inactivation should be investigated. For inactivation of different virus types, efforts should be directed toward finding the optimal conditions for effecting the most efficient destruction of infectivity while retaining the greatest degree of antigenicity. By selection of highly antigenic strains better immunogenic antigens should be found for incorporation in monovalent or polyvalent vaccines.

Different concentrations of aluminum gel should be compared in order to find the optimum amount of adjuvant. The effectiveness of other adjuvants should not be overlooked. Among adjuvants in use a water-in-oil emulsion has been tried successfully. McLENNAN *et al.* [11] have shown that by using a single batch of tetanus toxoid, in comparable amounts as a fluid vaccine (3 doses), as an aluminum salt preparation (2 doses), and as a water-in-oil emulsion (1 dose), the aluminum phosphate adsorbed toxoid induced approximately twice the average peak response as did the fluid vaccine, whereas emulsified toxoid gave a peak response later and about 10 times higher than that of the fluid preparation.

The effectiveness of a single dose of antigen in water-in-oil emulsion has been shown, both in animals with various antigens [5] and in man with influenza vaccine [8]. Providing that the local reaction after the injection of a proper dose of water-in-oil emulsified AHS vaccine in horses does not prove discouraging, the vaccine mixed with this adjuvant should be studied on a larger scale.

The duration of immunity after the second injection should also be investigated in a sufficient number of animals. In 2 horses under observation we have found detectable

amounts of neutralizing antibody 12 months after the second injection.

The immunogenic effectiveness of boosting with killed vaccine was also demonstrated. It remains necessary, however, to establish the schedule of immunization, number and interval of injections, composition, quality of antigens and adjuvant and finally the time for stimulation of immunity by a proper booster dose of vaccine.

*Acknowledgement*

For helpful support, the authors thank Dr. M. KAVEH, Director of the State Razi Serum Institute.

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