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African Horse-Sickness Live and Killed Virus Tissue Culture Vaccine (*)

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INTRODUCTION

Since Alexander [1] demonstrated that African horse sickness (AHS) virus could be attenuated in mice by serial intracerebral passages, mouse adapted neurotropic strains of the virus have been widely used for vaccine preparation.

The vaccine prepared by this method was proved to be superior to inactivated tissue vaccines prepared by various workers [3, 22, 23], not only because it was safer and more effective, but also because it was much cheaper than producing the vaccine by the other methods. The method, however, suffered from some disadvantages and thus other suitable hosts of the virus have been looked for and the necessity of developing a new technique for the production of large amounts of vaccine have been felt from the time of introduction of neurotropic mouse brain vaccine.

Embryonated eggs have been shown to be of potential value for vaccine production, although the original work carried out by Alexander did not give satisfactory results. [2]

Attempts to find a better host among tissue culture cells have been made by various workers. Mirchamsy and Taslimi [13] adapted an Asian strain of the virus to primary cultures of hamsters kidney cells. Erasmus [4] cultivated the virus in monolayers of chicken embryo fibroblast cells without cytopathic effects yielding rather low titers of the virus. Ozawa and Hazrati [16] demonstrated that the virus could be adapted to cell lines such as MS* [12] and BHK† cells, producing cytopathic changes, and that all neurotropic strains could be adapted to MS cells yielding high titers of the virus.

Adaptation of neurotropic strains of horse-sickness virus to the MS cells led to the development of vaccine produced in this new host system instead of mouse brains. The usefulness of several cell lines for horse sickness vaccine production, the technique of vaccine preparation and the factors essential for constant high yield of virus were studied by Ozawa, Hazrati and Erol. [17] The vaccine was used for the immunization of soliped animals by the same authors and others. [9, 14, 17, 18]

Inactivated tissue culture vaccine was also experimentally prepared by Ozawa and Bahrami. [19]

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*MS: Monkey Kidney Stable

†BHK: Baby Hamster Kidney

In this communication a summary of the results of experiments with these vaccines and the procedures for the production of these vaccines are presented.

1. Horse-Sickness Live-Virus Tissue Culture Vaccine

Tissue Culture. — Among different primary cell cultures established cell lines susceptible to the strains of horse-sickness virus, monkey kidney stable, cells developed by Doctor Kanda [12], were chosen for vaccine preparation because; (a) African horse-sickness virus, both viscerotropic and attenuated neurotropic strains, easily became adapted to the cells producing CPE from the first passage; (b) high titers were obtained and the incubation periods were shorter; (c) MS cell cultures were easy to prepare, the cells were stable and retained their healthy appearance.

After several passages, AHS virus produced complete CPE within two days in the cells, and in some cases almost 100 percent of the cells became detached from the glass surface within 24 hours after infection. The virus yield in such tissue culture fluids harvesed withtin one or two days after infection is usually very high.

A maximum titer of $10^{8.5}$ TCID50/ml. obtained in MS cells 48 hours after infection with a neurotropic strain of type 9, namely strain S2, is believed to be the highest titer yet reported with African horse-sickness virus. [14]

Vaccine Seed Virus. — The mouse adapted neurotropic vaccine strains attenuated according to the technique developed by Alexander [1], through more than 100 successive intracerebral passages in adult mice, were used as vaccine antigens. Seven of these strains, namely A501, OD, L. Vryheid, VH, 114 and Karen, representatives of type 1 to 7 respectively, were obtained from the Onderstepoort Veterinary Laboratories, South Africa, and the type 9 virus strain S2 was attenuated at the Razi Institute in Iran. [8]

A few additional passages of each strain, were made in mice to prepare fresh seed virus to adapt to MS cell cultures and the identity of virus adapted to cell cultures was confirmed by neutralization tests with homologous antiserums. [10]

Since the antigenicity of horse-sickness virus decreases if the virus passage levels in MS cells increase [18], passages of virus strains in MS cells should be minimized. It is, therefore, recommended that the maximum passage level of liquid vaccine seed virus be limited to the fifth or sixth passage.

Maintenance of High Titers. — From the results of a series of experiments on the stability of virus grown in MS cell cultures [18]. it appears that undiluted virus fluids containing 2 percent calf serum are stable at 4° C. and that freezing of the virus fluid has a detrimental effect on the infectivity of virus. This was common with the virus grown in MS cells, maintained in three different maintenance mediums. The freezing effect was more evident in the maintenance medium containing calf serum. Virus titers retained in frozen mouse brains were higher than those in frozen tissue culture.

It appears that during storage at 4° C the virus is more stable when the pH of the fluids maintained at 6.5 or higher and that pH values lower than 6.4 have a detrimental effect on the infectivity of the virus.

In freeze drying of the vaccine .the concentration of calf serum in maintenance medium should be limited to 2 percent or less, because infectivity of virus suspended in the medium containinig calf serum decreased markedly at -25° C. However, the virus withstood freeze drying very well if the virus fluid was diluted in the proper freeze drying diluent.

In the experiments for maintaining high qualities of freeze dried vaccine produced at the Razi Institute [9], it was found that in properly prepared batches of vaccine there was no decrease in titers during a storage period of six to 12 months at 4° C. These vaccines showed no significant loss of virus titer even after being kept at 37° C. for three to seven days. Virus titers of reconstituted vaccine were fairly stable when stored at 4° C.

Although no precise investigations have been made, the residual moisture content of the freeze dried vaccine has an effect on the storage quality and life of the virus.

In addition to adequate drying ,it is essential to vacuum seal the product.

Safety and Immunizing Potency of Vaccine. — Safety and potency of monovalent tissue culture vaccine was tested and compared with that of monovalent mouse brain vaccine of the same virus type, in a total of 12 horses. [9] These vaccinated and two non-vaccinated horses were closely observed. They were bled seven weeks after vaccination to determine antibody responses and challenged with homologous virulent virus on the following day.

In the same manner, as in the horse, the safety and potency of both tissue culture and mouse brain vaccine were tested in a total of eight donkeys.

The results obtained indicated that the tissue culture vaccine causes no obvious adverse local or general post-vaccinal reactions and produces sufficient immunity in vaccinated animals to protect them from infection with the homologous type of virulent horse-sickness virus. Moreover, the antibody response in animals vaccinated with monovalent MS tissue culture vaccine was comparable to that obtained in those vaccinated with mouse brain vaccine.

In another experiment with similar results to the above [18], the potency of both monovalent and polyvalent tissue culture vaccines were investigated by vaccinating eight susceptible healthy horses.

The laboratory tests of the vaccine were supplemented by a field trial on a larger scale [9]. Four hundred and fifty donkeys, 45 horses, and 50 mules were vaccinated with monovalent tissue culture vaccine which contained approximately 6.32 x 10^6 TCID50 of strain S2. No adverse effects due to vaccination were observed during a six-months' observation period.

2. **Horse-Sickness Killed-Virus Tissue Culture Vaccine**

Inactivated horse-sickness vaccine prepared by adding formalin to infected horse tissue emulsion have been experimentally used by various workers. [3, 22, 23] Their results showed that the immunity developed in animals inoculated with this vaccine was transient and the margin of safety was small. Moreover, the keeping qualities of the vaccine were poor.

In spite of these unsatisfactory results, the vaccine has been used from time to time on a large scale when or where a better vaccine was not available.

On the other hand, unfavorable post-vaccinal reactions has been reported among equine vaccinated with live virus polyvalent vaccine prepared from infected mouse brains. [6, 15, 20, 21] This and the fact that high infectivity titers were regularly obtained in tissue cultures led to the development of an inactivated tissue culture vaccine.

The inactivated vaccine could be prepared either from viscerotropic or neuro-

troipc type of horse-sickness virus cultivated in MS cell cultures. A vaccine was prepared using type 9 virus by Ozawa and Bahrami. [19]

To prepare the vaccine the seventh passage of both viscerotropic and neurotropic strains of type 9 virus in MS cells were used as the source of the vaccine. Tissue culture fluids containing virus were centrifuged at 1200 g. for 15 minutes. Formalin was added to the supernatant to make the final concentration 1:3,000 and the mixture was kept at 4° C. for two weeks, shaking it at least once a day.

It was found that the viruses were completely inactivated within five days in this manner. The vaccine was found safe when tested in eight horses. These horses were inoculated with challenge virus five weeks after vaccination. Most of the vaccinated horses had rectal temperatures between 39.1 and 40.5 within a few days and all became normal within one week after challenge. No signs of abnormality were observed during an observation period of two months. The control horse, however, died of horse-sickness nine days after injection.

The antibody responses of these horses were lower than those in horses vaccinated with live virus vaccine. The protecting quality of killed virus vaccine, however, could be improved by using high titer virus and adding an appropritae adjuvant to the vaccine.

DISCUSSION AND RECOMMENDATIONS

African horse-sickness live virus tissue culture vaccine produced at the Razi Institute by using MS cells and neurotropic attenuated strains of the virus were found to be safe and effective by experimental vaccination of equine and in field trials.

More than 450,000 doses of monovalent and 300,000 doses of polyvalent vaccine prepared by this method have been satisfactorily used since the introduction of this vaccine, and so far there have been no adverse effects similar to those observed with mouse brain vaccines.

The freeze dried live virus tissue culture vaccine keeps its quality fairly well under different storage conditions. It should, however, be stored in a refrigerator until immediately before use. Vaccine must be protected from direct sunlight and heat and be used within a few hours after being reconstituted. Alcohol, acids, or disinfectants must not be used for the sterilization of syringes. The dose is the same for all equine animals, regardless of age and size.

There is no danger of overdosing, but underdosing will interfere with successful immunization. Animals inoculated for the first time are expected to react occasionally between the seventh and the 11th day after inoculation.

Unfavorable post-vaccination reactions with an occasional loss of horses have been reported when using live virus polyvalent mouse brain vaccine in India and Israel. [15, 20, 21] These post-vaccinal reactions were even more serious in vaccinated donkeys. Severe reactions and a 4 to 10 percent mortality were reported from Cyprus and Pakistan among donkeys vaccinated with mouse brain polyvalent vaccine during the recent epizootic of the disease in the Middle East. [6] In India, type 2 virus was isolated from horses which died after vaccination.

Prior to these reports, type 7 (strain Karen) was recommended to be excluded from AHS virus polyvalent vaccine because of its virulence in guinea pigs. Thus the

extra virulence of some vaccine strains, the high susceptibility of some individual animals, the presence of mouse brain tissue, and even contamination of the vaccine with other viral and bacterial agents, could be suspected to be the cause of occasional severe post-vaccination reactions.

Another disadvantage of the mouse brain vaccine is that the production of the vaccine depends on the breeding, handling and controlling of the mice; it requires large numbers of mice and it is a rather tedious job to harvest infected mouse brains.

The newly developed tissue culture vaccine, on the other hand, has the advantage of being easier and less expensive yet giving a high quality of vaccine.

In addition, if MS cells are used as the host of virus, the specificity of each vaccine strain is tested readily in MS tube cultures and chances of contamination with other viral, fungal or bacterial organisms may be reduced. Moreover, if animals are rested after vaccination, post-vaccination reactions with these tissue culture vaccines are almost unrecognizable, because these vaccines contain very small amounts of foreign protein and lipids.

Tissue culture vaccine produces adequate immunity in vaccinated animals to protect them from infection with the homologous viscerotropic virus. Antibody response in horses and donkeys vaccinated with monovalent vaccines were found to be high and the immunity in these animals was solid.

Horses vaccinated with polyvalent vaccine often failed to develop specific antibodies to certain components of the vaccine. This may have been due to poor antigenicity of the respective component as Howell [11] and Mirehamsy [14] suspected or to interference between certain virus types incorporated in the vaccine.

Since the use of polyvalent vaccine does not assure the development of immunity against all vaccinal components, the use of monovalent AHS vaccine seems preferable in regions where only one virus type exists naturally. Theoretically, the use of polyvalent live virus vaccines in such areas may even be hazardous. New virus types may arise by genetic recombination after simultaneous inoculation of various attenuated strains into susceptible hosts. Fortunately, the experience gained in certain Middle East countries in recent years clearly indicated that this danger can be neglected.

Although it is not practical to use the inactivated horse-sickness tissue culture vaccine on a large scale, it has advantages on some particular occasions.

It is better to employ this vaccine when equines have to be shipped from countries having epidemics to disease-free areas or vice versa. When vaccinating very valuable animals ,a first vaccination with inactivated vaccine is more desirable.

Procedures for the production of African horse-sickness live-virus tissue culture vaccine. — All known types of AHS virus attenuated by 100 intracerebral passages in mice, are adapted to MS cells or any other cells equivalent to MS cells. Supernatant fluids of 10 percent brain suspensions prepared in maintenance medium are used as inoculums. Just before cell sheets are formed in bottle cultures the nutrient medium is drained and the virus is allowed to be absorbed for about one hour at 37° C.

The inoculum is then drained and fresh maintenance medium is added to each bottle culture. It usually takes four to eight days to produce cytopathic changes in the first passage. Sub-passages of virus are made whenever cytopathic changes become widespread. If cytopathic changes are not evident in the first passage, blind passages are made seven to eight days after infection. The pH vaules of the maintenance medium

should always be kept higher than 6.4.

The third passage in tissue cultures of vaccine strains is freeze-dried. For freeze-drying ,the virus fluid is diluted at least three times in sterilized lactose-peptone-tris-buffer solution. The virus fluid dispensed in sterilized small bottles or ampoules is freeze-dried, and each container is sealed under vacuum. Freeze-dried antigen may be stored at 4° C or lower temperature. It is recommended to prepare fresh freeze-dried antigen every 24 months.

Frozen stock antigen is not absolutely necessary but as a precautionary measure some of the virus fluid prepared for freeze-drying can be dispensed in suitable small containers and stored at -20° C.

Supernatant virus fluids containing 2 percent calf serum of the fourth and fifth passage in tissue cultures are dispensed in small containers fitted with rubber stoppers, and stored at 3° to 4° C. The final pH value of the fluids should be between 6.5 and 7.5. Fresh liquid stock seed virus should be prepared at least every six months.

Supernatant virus fluids of the fifth and sixth passages in tissue cultures are dispensed in suitable containers and stored at 3° to 4° C. To maintain high titers, fresh vaccine seed virus should be prepared at least every three months. All the types of vaccine seed virus should be able to destroy monolayers of MS cells within 48 hours after infection.

For the preparation of vaccine, using large flat bottles, cell cultures are prepared. For MS cells, YLE containing 5 percent inactivated calf serum is used as the growth medium. The density of the cells over the entire surface of the bottles should be even.

Just before complete cell sheets are formed, the growth medium is discarded. The cells are infected with liquid vaccine seed virus, the volume of which is approximately 1/100th the volume of the growth medium used. The bottles of infected cultures are rocked a few times during the adsorption period.

Usually, viruses are allowed to adsorb on MS cells for two hours at 37° C. Fresh maintenance medium containing 1 to 2 percent inactivated calf serum is placed in each bottle and the cultures are incubated at 37° C.

In MS cell cultures, complete CPE usually appears within two days after infection. Some strains such as tyep 6 and 9 produce complete CPE wthin 24 hours after infection. To obtain the highest titer, virus fluids should be harvested when almost all cells remaining on the surface of the glass have a distinct CPE. After shaking vigorously, virus fluids are harvested and stored at 4° C.

Virus fluids at 4° C. are centrifuged in a refrigerated centrifuge at approximately 1,000 g. for ten minutes. The supernatant fluid is harvested paying attention to avoid contamination of large cell debris.

For monovalent vaccine, usually one volume of the supernatant virus is mixed with ten volumes of chilled freeze-drying diluent* and nine volumes of chilled distilled water.

For polyvalent vaccine usually N volumes of virus fluids of vaccine strains mixed in eqaul parts are mixed with ten volumes of the chilled freeze-dryinv diluent and (10-N)

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*Five hundred milliliters of aqueous solution containing lactose 100 mg. and peptone 20 gm. is mixed with an equal volume of tris buffer (pH 7.4.)

volumes of chilled distilled water, where N is the number of strains incorporated in the vaccine.

The total volume of virus fluid and distilled water added is always equal to the volume of freeze-drying diluent containing penicillin and streptomycin. All these materials and procedures should be kept under aseptic conditions.

If one wants to dilute any strain of virus more than 100 times, it is safer to store the supernatant virus fluid at 4° C., adjusting the pH value between 6.5 and 7.0 and determining the titer of virus in the fluid. If the titer is $10^{7.5}$ TCID50 per ml., the virus fluid may be diluted 200 times in diluents for freeze-drying.

Diluted liquid vaccine may be stored at 4° C. or lower temperature until freeze-dried.

The liquid vaccine is dispensed in sterilized ampoules or bottles and freeze-dried. Each ampoule or bottle should be vacuum sealed. The freeze-dried vaccine should be a white or light pink pellet that should maintain its shape during storage in a refrigerator.

Stock seed virus, preferably liquid stock seed virus, should be tested for specificity, antigenicity and safety. Specificity may be tested by neutralization tests in tube cultures. The neutralized cultures should be observed for ten days after infection to insure that there is no other virus mixed within the seed virus.

Antigenicity and safety may be tested in horses simultaneously. Development of specific antibodies in the serum of the horses inoculated approximately four weeks previously with each attenuated strain should be confirmed by neutralization tests in tissue cultures. After injection, the horses are kept under close observation for four or five weeks and temperatures are recorded daily. If they are kept under comfortable conditions without work, they should not develop any severe signs of abnormality.

The use of guinea pigs in determining the potency and antigenicity of attenuated strains has been recommended by Erasmus.[5]

Control cell cultures used for the production of liquid vaccine must be kept at 37° C for at least two weeks after the preparation of cell cultures in order to detect any virus or microorganisms accidentally introduced with the medium. If any abnormality is found in the control cell cultures, the medium should be tested by subcultivations.

Virus fluids harvested in suitable containers must be carefully examined for bacterial or fungus contamination before pooling and preparation of liquid vaccine.

The sterility of freeze-dried vaccine picked up randomy from each batch is tested by inoculating 1.0 ml. of reconstituted vaccine into suitable media such as thioglycollate and incubating for seven days at 37° C. If there is contamination, the contaminant is identified and the test repeated. Ten adult mice are inoculated intraperitoneally, each with 0.2 ml. of reconstituted vaccine of each batch and kept under observation for two weeks to prove that the vaccine is not pathogenic by this route of injection. It is recommended to test the safety of each or combined batches of freeze-dried vaccine in non-immunized horses keeping them under close observation for one month after inoculation.

Two bottles of freeze-dried vaccine are placed in the incubator at 37° C. and after three days the incubated vaccine is titrated in tissue culture in comparison with vaccine stored at 4° C. The difference in titers should be less than 0.5 log.

The minimum requirements for African horse-sickness virus vaccines are approxi-

mately 20,000 TCID50 per dose for monovalent vaccine and approximately N x 20,000 TCID50 per dose for polyvalent vaccine; N is the number of strains incorporated. All vaccine vials should be tested for vacuum before they are issued.

It is recomemnded to test from time to time the malignancy of cells used for producing vaccine by the method described by Foley and others.[7] Tested stock cell lines may be kept frozen ,and cultivation of vaccine strains of virus can be done in the cells within a limited number of passages.

SUMMARY

Monkey kidney stable (MS) cell line has been used as the host of African horse-sickness virus, and both monovalent and polyvalent live virus tissue culture vaccines were produced. These vaccines have been tested in the laboratory and in the field.

It appears that these vaccines are as good as or even better than mouse brain vaccine. There is little or no post-vaccination reaction among equines inoculated with tissue culture vaccines. The antibody response in horses and donkeys inoculated with tissue culture vaccine was comparable to that obtained with attenuated mouse brain vaccines.

African horse-sickness killed virus tissue culture vaccine was experimentally used for immunizing horses. Inactivated vaccine prepared either from viscerotropic or neurotropic type 9 African horse-sickness virus produced antibodies. Immunity developed in all horses vaccinated with various amounts of the vaccine, and protected them from infection when challenged five weeks after vaccination.

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