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# ATTEMPTS TO VACCINATE FOALS WITH LIVING TISSUE CULTURE ADAPTED HORSE SICKNESS VIRUS

by

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It is well known that horses may be vaccinated successfully with mouse-adapted types of Horse Sickness virus (HSV), as described by Alexander (1).

During the recent outbreak of African Horse Sickness in the Middle-East region, the Razi Institute, Iran was able to produce, with the collaboration of Onderstepoort Laboratory and under the auspices of F.A.O., large amounts of polyvalent mouse-adapted virus-vaccine to combat the disease in the region (9). It was felt, however, that the immunization schedule might be simplified by using a modified tissue culture-adapted virus, if a long-term mass vaccinalion programme were to be carried out in the infected countries. This paper gives an account of the techniques developed for the production of living attenuated vaccine in tissue culture.

## MATERIALS AND METHODS

### I. — ADAPTATION OF VIRUS TO TISSUE CULTURE

1) *Virus origin.* — First, we adapted the local strain n° 28 of HSV to hamster kidney cells (HKC) (7). This strain was isolated in 1960 from blood collected in Khorram-Abad (Iran) from a horse which was showing symp-

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toms of the acute cardiac form of the disease. After four passages in baby mice, the virus was passaged 23 times in HKC, as previously described.

The primary adult hamster kidney cells used in our earlier investigation for adaptation and for growth of HSV was replaced in this study by primary baby hamster kidney cells. The kidney cells of baby hamsters less than a week old provide a regular monolayer sheet and a higher titre of strain n° 28 of HSV when it was serially transferred.

Two subcultures of the virus were passed 10 times in monkey kidney cells line (MS) or in hamster kidney cells line (BHK 21) starting from 8th passage in HK.

Adaptation of HSV to MS was first achieved in this Institute by Ozawa and Hazrati (8).

The second virus used in this study was strain S 2 which was isolated also in 1960 in Shiraz (Iran). This strain was successfully attenuated by successive intracerebral passages in adult mice by Hazrati and Taslimi (4). The authors showed that, after 65 intracerebral passages, this virus was successfully attenuated; after 100 passages, it had completely lost its viscerotropic character and it is being used as a neurotropic virus-vaccine. By cross-neutralization tests in MS tissue culture, we have found that these two virus are antigenically identical with a third local strain S 10/60 which is used in this study as a virulent virus for challenge of immunized foals. The latter strain had been classified by Howell (5) as a new group n° 9 of Onderstepoort.

In this study, S 2 virus was passaged ten times in BHK 21 or in MS cells. In both cases, the adaptation was stable and in the second passage, a marked CPE, usually seen as granulation, rounding and lysis was noticed. The log. TC ID 50/ml of these 2 subcultures at the 10th passage were 6.5 (MS) and 6.26 (BHK 21) and the log. LD 50/ml mice was between 6.5 and 7.

2) *Culture media.* — The culture medium for MS was L.A.Y.E. medium of Franklin, Rubin and David (3). Ten percent pooled heated cattle serum was added. The same medium with 5 percent cattle serum was used as a maintenance medium.

BHK 21 was grown in Eagle's modified medium with tryptose phosphate broth 10 percent and calf serum 10 percent, as described by MacPherson and Stoker (6). This medium was replaced later by a medium containing 0.5 percent lactalbumine hydrolysate in Hank's saline. The maintenance media were identical with the growth media. The culture medium for baby hamster kidney cells was CSV 5 (2) containing 20 percent calf serum and the maintenance medium was the same but contained 10 percent calf serum instead of 10 percent normal horse serum as used in our previous investigation.

For virus passage or sero-neutralization tests, screw-capped 16 × 125 mm "Pyrex" tubes were used. MS cells usually formed complete monolayer sheets in 2-3 days. In the case of BHK 21, the sheet was formed in 3-4 days. All media had a final pH of 7.1-7.2: 100 units penicillin, 100 g. streptomycin and 100 units mycostatin per ml were added before use.

3) *Storage of virus.* — All subcultures were stored at — 40 °C. In order to study the viral infectivity during storage, 1 percent gelatin or 5 percent sucrose was mixed with separate lots of virus before freezing. The final pH which was still 7.1-7.2 was not changed. To investigate the stability of antigens, one part of the antigen used for the inoculation of the foals was lyophilized and kept at — 20 °C. Samples of various preparations stored at — 40 °C with or without additives as well as lyophilized virus were tested in mice at monthly intervals for infectivity or were titrated in tissue culture. It was found that the frozen preparations did not retain the initial titres in mice or in tissue culture, but that the titres were reduced by 2 to 3 log. after 2 months storage at — 40 °C. In some cases, a sudden fall in titre was found but the titres were however more stable when additives had been added before storage at — 40 °C.

The lyophilized preparations retained the initial titres after lyophiliziation, even 9 months after storage at — 20 °C.

4) *Experimental animals.* — Foals, between the ages of 12 and 24 months, were obtained from areas where no previous outbreak of the disease had been recorded.

The sera of all foals were screened before incubation as well as 4 weeks after vaccination in order to measure the neutralizing antibody. It was found that, before vaccination, the sera of all the foals were free from antibodies.

5) *In vitro sero-neutralization tests.* — Sera were inactivated at 56 °C for 30 minutes. Fourfold dilutions from 1 : 4 to 1 : 1024 were made of each serum. Two-tenths ml of each serum dilution were mixed with 0.2 ml of the test dose of virus and the mixture was incubated for 2 hours at room temperature. The test dose of virus was that previously calculated to contain 100 TCID 50 of lyophilized virus. Controls of normal and hyperimmune horse serum, as well as a virus titration were included in each test.

After incubation, 0.1 ml of each serum-virus mixture was inoculated into two tissue culture tubes and incubated at 37 °C. The tubes were read each day for cytopathic effect and the readings on the day that the control virus titration showed about 100 TC ID 50 of virus to be present were used in the final readings. Complete protection of any MS culture was regarded

as evidence of the presence of antibody.

TABLE 1- INFECTIVITY OF HSV BY SERIAL TRANSFERS IN ADULT AND IN BABY HAMSTER KIDNEY CELLS.

| PASSAGE NO | LOG ID 50/ML OF COLLECTED FLUID AFTER 96H. | |
|---|---|---|
| | ADULT CELLS | BABY CELLS |
| I | 4 2 | 5 72 |
| II | 5 0 | 6 5 |
| III | 6 2 | 7 65 |
| IV | 6 14 | 7 97 |
| V | 5 08 | 7 08 |
| VI | 6 22 | 7 40 |
| VII | 4 62 | 6 20 |
| VIII | 5 18 | 5 12 |
| IX | 4 07 | 6 35 |
| X | 5 25 | 6 55 |
| XI | N T | 5 0 |
| XII | N T | 5 25 |

N.T = NOT TESTED

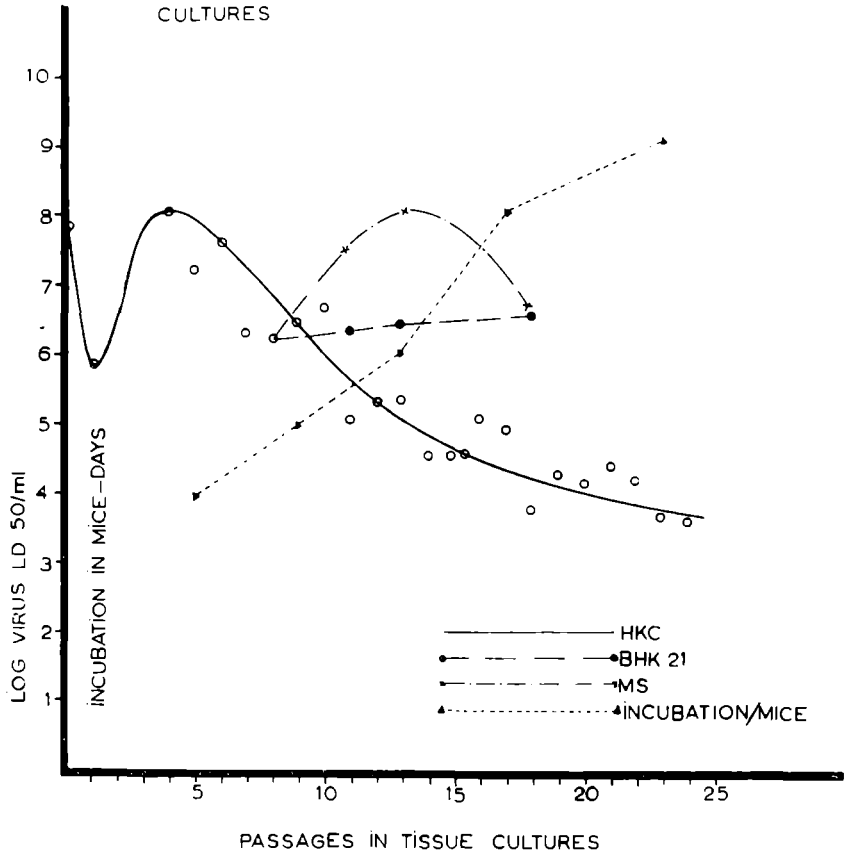
All serum titres are expressed as the reciprocal of the highest dilution of serum that completely inhibited cytopathogenic effect in both tubes inoculated.

6) *Cntrol of infectivity of virus in mice.* — The infectivity of the cultures was tested in mice. A volume of 0.03 ml of each dilution of virus was injected intracerebrally into each of a group of 8-10 adult mice. Mortality were recorded daily and the end-points determined after 2 week's observation, by using the Reed and Muench method (10).

7) *Challenge of immunized foals.* — The immunity of all foals was challenged by the intravenous inoculation, usually 8 weeks after they had received tissue culture-adapted virus, of 1 ml of 6-8 percent brain suspension of baby mice which had been injected with virulent strain S 10/60 and killed while *in extremis.* The suspension of virulent virus was centrifuged

for 15 minutes at 2,500 r.p.m. at 2 °C before inoculation. The challenge dose contained $10^7$ to $10^{7,5}$ LD 50 ml/mice.

FIG 1 THE INFECTIVITY OF VIRULENT HORSE SICKNESS VIRUS FOR MICE AFTER SERIAL TRANSFERS IN DIFFERENT TISSUE CULTURES




## II. — RESULTS

The virus yields in adult and in baby hamster kidney cells are compared in Table I. It is evident that in baby hamster kidney cells the virus titre is higher than in adult hamster kidney cells. The pathogenicity for mice of the virulent strain n° 28, adapted to the HK, MS and BHK 21, is shown in Fig. 1.

TABLE 2-IMMUNITY IN FOALS INOCULATED WITH VIRULENT LOCAL STRAIN NO 28 OF HSV AFTER 8, 13 AND 23 PASSAGES IN BHK

| ANIMAL NO | LD 50/ MICE | PASSAGE LEVEL | MAXIMUM TEMPERATURE C° | DURATION OF PYREXIA (DAYS) | TITRE | REACTION TO CHALLENGE | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | MAX. TEMP. C° | DURATION OF PYREXIA (DAYS) | |
| 1 | $10^{6.12}$ | HK 8 | 40.0 | 7 | 1024< | 40.4 | 8 | |
| 2 | " | " | 40.1 | 8 | 1024< | 40.1 | 8 | |
| 3 | $10^{5.30}$ | HK 13 | 39.6 | 14 | 256 | N | 5 | |
| 4 | " | " | N | | 64 | 41.5 | 7 | |
| 5 | $10^{4.51}$ | HK 23 | N | | 4> | 41.0 | 6 | |
| 6 | " | " | N | | 4> | 40.2 | 5 | |
| 7 | $10^{5.51}$ | " | N | | 4 | 40.0 | 6 | |
| 8 | " | " | N | | 4> | 40.8 | 10 | |
| 9 | $10^{5.69}$ | " | N | | 4 | N | | |
| 10 | " | " | N | | 4> | N | | |
| 11 | $10^{5.91}$ | " | N | | 4 | 40.4 | 7 | |
| 12 | CONTROL | | | | 4> | 40.7 | | DIED |
| 13 | // | | | | 4> | 41 | | DIED |

N=NORMAL TEMPERATURE HK=BABY HAMSTER KIDNEY PRIMARY CELLS

TABLE 3-IMMUNITY IN FOALS INOCULATED WITH VIRULENT LOCAL STRAIN NO 28 OF HSV AFTER 8 PASSAGES IN HK AND 10 PASSAGES IN MS OR IN BHK CELLS LINES

| ANIMAL NO | VIRUS LD50/MICE | PASSAGE LEVEL | MAXIMUM TEMPERATURE C° | DURATION OF PYREXIA (DAYS) | TITRE | REACTION TO CHALLENGE | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | MAX. TEMP. C° | DURATION OF PYREXIA (DAYS) | |
| 14 | $10^{8.10}$ | HK8 MS5 | 39.6 | 11 | 1024< | N | | |
| 15 | " | " | N | | 1024 | 41.5 | 4 | |
| 16 | $10^{6.7}$ | HK8 MS10 | 40.0 | 4 | 1024 | N | | |
| 17 | " | " | N | | 1024 | N | | |
| 18 | $10^{6.50}$ | HK8 BHK10 | N | | 64 | | | |
| 19 | " | " | N | | 1024< | | | |
| 20 | CONTROL | | | | 4> | 41.2 | | DIED |

N=NORMAL TEMPERATURE

TABLE 4 - IMMUNITY IN FOALS INOCULATED WITH NEUROTROPIC STRAIN S.2 OF HSV AFTER 100 INTRACEREBRAL PASSAGES IN MICE AND 10 PASSAGES IN MS OR IN BHK CELLS LINES

| ANIMAL NO | VIRUS DOSE LD50/MICE | PASSAGE LEVEL | MAX. TEMPERATURE C° | DURATION OF PYREXIA (DAYS) | TITRE | REACTION TO CHALLENGE | |
|---|---|---|---|---|---|---|---|
| | | | | | | MAX. TEMP. C° | DURATION OF PYREXIA DAYS |
| 1 | $10^{6}$ | 100 MICE | 39 7 | 2 | 1024 < | N | |
| 2 | " | " | 40 0 | 4 | 1024 < | N | |
| 3 | " | " | N | | 256 | 40 2 | 5 |
| 4 | " | " | 41.2 | 5 | 256 < | N | |
| 5 | " | " | 39.6 | 3 | 1024 | 40 7 | 4 |
| 6 | $10^{6.5}$ | 10 MS 100 MICE/- | 40.1 | 4 | 1024 < | N | |
| 7 | " | " | N | | 1024 < | N | |
| 8 | $10^{6.26}$ | 10 BHK 100 MICE/- | N | | 256 | N | |
| 9 | " | " | N | | 64 | N | |
| 10 | CONTROL | | | | 4> | 41 | DIED |

N=NORMAL TEMPERATURE

It is worth mentioning that, by serial transfers of this viscerotropic strain, there was first an increase and then a regular decrease in the infectivity for mice. After 23 passages in HK, the LD 50 ml/mice was about $10^{-3.55}$. On the other hand, the two subcultures of this virus, when passed 10 times in MS cells after 8 passages in HK, showed an increase and then a decrease in pathogenicity for mice. The LD 50/ml of 10th passage in MS was $10^{-6.7}$ and in the BHK 21 it was $10^{-6.5}$.

The immunizing quality of these subcultures is shown in Tables II and III. After 8-13 passages in HK, when the virulence for mice started to decrease, the inoculated foals showed a rather mild temperature reaction and a high neutralising-antibody titre, and resisted challenge with homologous virulent virus.

After 23 passages of virulent virus in HK, the LD 50/ml for mice was reduced to $10^{-3.55}$, and the incubation period for mice increased from four days for the original virus to nine days. The foals vaccinated with this passage level did not show any general reaction and all resisted challenge with the homologous virulent virus. The absence of neutralizing antibody shows, on the other hand, the poor antigenic quality of the virus after 23 passages in HK.

The results of the vaccination of foals with the viscerotropic virus passaged 8 times in HK and then 5-10 times in MS or in BHK 21 cells lines are given in Table III. The MS subculture produced a high neutralizing antibody. The antibody obtained by using BHK 21 subculture was 4-16 fold lower, but both subcultures protected the immunized foals agains challenge with virulent virus.

In order to study the safety of the modified virus used in these experiments, the temperatures of all the vaccinated animals were recorded twice a day. Careful clinical examinations for a period of 8 weeks after immunization have not disclosed any untoward reaction. Finally, the blood of the vaccinated animals during the stage of pyrexia failed to induce the disease in normal foals or in mice.

From these data, it may be assumed that this virulent virus, after adaptation to HK or after passages in MS or in BHK cells lines, lost its viscerotropic nature.

The results of immunization of foals with the neurotropic strain S 2, which was passaged 10 times in MS or in BHK 21 cells lines, are shown in Table IV. The sera of the animals injected with MS subculture had a higher neutralizing titre; the animals showed a mild thermal reaction and resisted challenge with the homologous virulent virus.

In another experiment, an account of which will be published later, neurotropic strains of all the previously known 7 types of HSV were easily adapted to MS cells. After 10 passages, the log. LD 50/ml for mice and the log. TC ID 50/ml for MS cells line were between 6 and 7.

Foals were immunized with a lyophilized mixture of equal quantities of these types. Neither local nor general reactions were noted and all the foals had sufficient amounts of neutralizing antibody at four weeks following inoculation when tested with the homologous type virus.

## SUMMARY AND CONCLUSIONS

1) The virus of horse sickness grown on primary kidney cultures of *young* hamsters has regularly a higher titre than virus grown on primary kidney cultures of *adult* hamsters.

2) A new method is being used for serum-neutralisation of the virus of horse sickness grown in tissue cultures. This method may replace, with advantage, the long and expensive method in which white mice are used.

3) In this note, there is given an account of the tissue culture technique used to modify the virulence of the virus of horse sickness, type 9, and to estimate the extent to which it has been modified.

4) An increase followed by a decrease in infectivity of the virus for white mice is found in successive passages in primary kidney cultures of young hamsters.

After 8-13 passages, inoculated foals show a slight rise in temperature and quite a high titre of neutralising antibodies : they also withstand challenge by the virulent homologous virus.

After 23 passages, the infectivity of the virus for white mice decreases considerably and the incubation period increases from 4 to 9 days.

It has been shown that foals inoculated with this passage of the culture virus have only a low antibody titre, but they withstand challenge by virulent homologous virus.

5) The different neurotropic types of the virus of horse sickness can easily be adapted to tissue cell cultures, instead of with vaccine prepared with infected brain of white mice.

## RESUME ET CONCLUSIONS

1) En cutlure primaire de rein de jeune hamster, on peut obtenir régulièrement une culture de virus de la peste équine d'un titre plus élevé qu'en culture de rein de hamster adulte.

2) Une méthode nouvelle a été mise au point pour la séroneutralisation du virus de la peste équine en culture de tissus. Cette méthode peut avantageusement remplacer la méthode longue et coûteuse de séro-neutralisation sur souris albinos.

3) Cette note donne un compte rendu de la méthode de culture de tissu mise au point pour modifier la virulence d'une souche de type 9 du virus de la peste équine et pour évaluer son degré d'atténuation.

4) Par des passages successifs en culture primaire de rein de jeune hamster, on observe d'abord une augmentation puis une diminution du pouvoir infectieux du virus pour la souris albinos.

Après 8 à 13 passages, les poulains inoculés montrent une légère hyperthermie, un titre en anticorps neutralisants assez élevé et ils résistent à l'épreuve avec le virus virulent homologue.

Après 23 passages, le pouvoir infectieux pour la souris albinos diminue considérablement et la durée de l'incubation de la maladie chez cet animal

passe de quatre à neuf jours.

Il a été montré que des poulains, inoculés avec ce degré de passage du virus en culture, ont un taux d'anticorps très faible mais résistent à l'épreuve avec le virus virulent homologue.

5) Les différents types de virus neurotropes de la peste équine peuvent être aisément adaptés aux lignées cellulaires MS ou BHK.

Les chevaux peuvent être vaccinés avec ces virus adaptés aux cultures cellulaires à la place de vaccin préparé à partir du cerveau infecté de la souris albinos.

* * *

## RESUMEN Y CONCLUSIONES

1) En cultivo primario de riñón de hamster joven, se puede obtener regularmente un cultivo de virus de la peste equina de título más elevado que en cultivo de riñón de hamster adulto.

2) Se ha puesto a punto un método nuevo para la seroneutralización del virus de la peste equina en cultivo de tejidos. Dichométodo puede sustituir de manera ventajosa el largo y costoso método de seroneutralización en ratones albinos.

3) Esta nota proporciona una reseña del método de cultivo de tejido puesto a punto para modificar la virulencia de una cepa de tipo 9 del virus de la peste equina y para valorar su grado de atenuación.

4) Por pases sucesivos en cultivo primario de riñón de hamster joven, se observa primeramente un aumento y después una disminución del poder infeccioso del virus para el ratón albino.

Después de 8 a 13 pases, los potros inoculados muestran una ligera hipertermia, un título de anticuerpos neutralizantes bastante elevado y resisten a la prueba con el virus virulento homólogo.

Después de 23 pases, el poder infeccioso para el ratón albino disminuye considerablemente y la duración de la incubación de la incubación de la enfermedad en dicho animal pasa de 4 a 9 días.

Se ha demostrado que potros, inoculados con este grado de pase del virus en cultivo, tienen un índice de anticuerpos muy reducido pero resisten a la prueba con el virus virulento homólogo.

5) Los diferentes tipos de virus neurótropos de la peste equina se los puede adaptar fácilmente a las líneas celulares MS o BHK.

Los caballos pueden ser vacunados con estos virus adaptados a los

cultivos celulares en vez de vacuna preparada con cerebro infectado de ratón albino.

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## REFERENCES

(1) ALEXANDER (R. A.). — *Onderstepoort J. vet. Sci.,* 1935, 4, 349.
(2) COOPER (P. D.), WILSON (J. N.) and BURT (A. M.). — *J. gen. Microbiol.,* 1959, 21, 702.
(3) FRANKLIN (R. M.), RUBIN (H.) and DAVIS (C. A.). — *Virology,* 1957, 3, 96.
(4) HAZRATI (A.) and TASLIMI (H.). — *XVIIth World vet. Congr., Hanover,* 1963, 1, 535.
(5) HOWELL (P. G.). — *Onderstepoort J. vet. Res.,* 1962, 29, 139.
(6) MAC PHERSON (I. A.) and STOKER (M. G. P.). — *Virology,* 1962, 16, 147.
(7) MIRCHAMSY (H.) and TASLIMI (H.). — *Nature,* 1963, 198, 704.
(8) OZAWA (Y.) and HAZRATI (A.). — Personal communication, 1963.
(9) RAFYI (A.). — *Arch. Inst. Hessarak,* 1961, 13, 60.
(10) REED (L. J.) and MUENCH (H. A.). — *Amer. J. Hyg.,* 1938, 27, 493.