Arch. Inst. Razi, 1973, 25, 23 - 46

A REVIEW ON AETIOLOGY AND PATHOGENY OF AFRICAN HORSESICKNESS. (*)

By

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Introduction.

African horsesickness (AHS) is among the world's most fatal infectious diseases of equines which is caused by an insect-borne virus with 9 different immunological types. The disease, in its acute (pulmonary) and subacute (cardiac) forms is characterized by marked clinical symptoms, high morbidity and high mortality. Recovery is observed mostly in the mild form (horsesickness fever) of the disease.

AHS has been known as a meridional sickness of Africa for many centuries. When European settlers started to move to South Africa, their imported horses and mules lost because of this disease.

During last two centuries severe outbreaks of AHS were observed at irregular intervals in South Africa with large loss in 1780,1801,1839,1854,1862, 1891,1914,1918,1923,1940,1946, and 1953.

A report from Henning (29), recorded a great loss of equine imported by Dutch East India Company in the cape of Good Hope at the beginning of eighteen century. The disease has been also existed in Central Africa for a long time.

AHS was, later on, directed to East Africa, in the direction of Red Sea and infected Yemen (1930), Palestine (1944), and Egypt (1958). In the summer of 1959 South Eastern regions of Iran, and during the spring of the following year all over Persian Gulf areas, became infected. In the same time the outbreak was reported from Afghanistan and Pakistan, and rapidly spread to India and most of the Middle Eastern countries. During the period of 1959-1961, by a devastating outbreak of the disease, this region lost over 300,000 of its equine.

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* This paper was originally prepared in English for Tribuna Veterinaria (Madrid) and its Spanish translation was published in Tribuna Veterinaria Nos.86,87,88 (1972).

In 1965, the disease was observed in Lybia, Tunisia, Algeria, and Morocco, and subsequently spread to Spain, and caused a lot of mortalities (27).

African horsesickness was at first not recognized as a specific disease. Many of investigators, because of the similarity of its clinical manifestation with anthrax and piroplasmosis, considered it to be identical with these diseases. Some others related it to human malaria or even thought to be caused by a fungus.

M'Fadyean (1900), Theiler (1901), and Nocard (1901) were the firsts who have shown the presence of the viral agent in the blood of infected horses and were able to transmit the disease to susceptible animals by filtered infected materials.

Preliminary investigations indicated that the virus is existed in the blood of infected animals, in a high concentration, during the febrile reaction. The virus is attached to the red blood cells (92), and for the same reason, it is present in all internal organs. More precise studies on the characterization of the virus have been done since the adaptation of the virus in laboratory animals was developed. The results of these studies are summarized in this review.

Physico-chemical properties.

Structure and physico-chemical constants.

M'Fadyean (1900), Nocard (1901), Sieber (1911), and Alexander (1935)(5), demonstrated the filtrability of AHS virus through Berkfeld and Chamberland filters or through Seitz EK filter pad.

Polson (1941) (78), studying the particle size of 6 different strains of AHS virus, reported a mean diameter of 50 mμ and 45.4 mμ for the virus particle, as determined by ultrafiltration and by ultracentrifugation methods, respectively, and stated that there was no significant difference in the size of virus particles of antigenically different used strains.

In a further studies Polson and Madsen (1954) (80), reported that tissues infected with neurotropic AHS virus strains contained at least two infective particles of 31.2 mμ and 50.8 mμ, and a non infective but antigenic component of 12 mμ in diameter. The latter particle, a soluble antigen possessing the complement fixing power, was found to remain in the supernatant fluid after ultracentrifugation of the virus suspension at 30.000 rpm for 2 hours.

Investigations by electron microscopy have shown the size of the virus being 40 to 80 mμ (12,31). This was confirmed by Polson and Deeks (1963)(81),

who succeeded on purification of AHS virus for electron microscopy by using the polyethylene glycol precipitation technique in combination with ultracentrifugation and zone electrophoresis, and reported that the virus, so purified, appeared to have diameter of 70 to 80 mμ with 92 rod-shaped subunits radiating from a spherical body.

Ozawa et al (1965,1966) (64,70), by electron microscopic investigation, estimated the size of virus being 45 to 75 mμ.

In a more recent studies, Oellermann et al. (1970) (60) found that AHS and Bluetongue viruses were morphologically similar, Both viruses were shown to possess a characteristic icosahedral shape, measure approximately 55 mμ in diameter. Furthermore, their capsids seemed to consist of only one layer which was constructed of hexamerpentamer arrangements of structural units giving a total of 32 capsomers. The viruses had a pseudo-envelopes which was removed by purification procedures without loss of infectivity. Basing on the absence of envelope and resistance to ether, the authors proposed to remove the viruses from arbovirus.

Attempts have been made to demonstrate the infectivity of AHS virus nucleis acid, by using the cold phenol technique of Gierer and Shramm (1956)(22), and by employing the same method in the presence of either EDTA (1), or 1% SDS (11). The nucleic acid thus obtained was also precipitated by means of cold ethanol to yield a more concentrated starting material. The preparation was immediately inoculated into suckling mice or onto MS cell sheets, pretreated by hypertonic salt solutions. The infectivity which observed in some instances, could possibly be attributed to the presence of intact virus, and it should be claimed that the attempts have so far failed to demonstrate the infectivity of viral nucleic acid (Kamali and Mirchamsy-unpublished data).

Consequently for obtaining information about the type of nucleic acid of AHS virus the susceptibility of virus multiplication to actinomycin D was tested (Mirchamsy and Taslimi 1966) (49). A moderate inhibition with actino mycin D at 0.1μ.g/ml., and a marked inhibition at 0.5 μg./ml. in cell culture-was observed. Ozawa (71), on the contrary, reported that 1.8 μg. actinomycin D had no effect on the growth of AHS virus in vero cells. Oellermann et al. (1970), (60) using BHK21 cell line, confirmed the findings of Mirchamsy and Taslimi and showed that AHS virus was sensitive to the effect of actynomycin D at a concentration of 0.05 μg./ml. They also clearly demonstrated the lack of inhibition of the replication of AHS virus by BUDR at 30 μg./ml. and confirmed the fact that AHS virus contains RNA as its genetic material.

By means of sucrose gradiant sedimentation Oellermann et al. (60) have shown the possibility of resolving the AHS virus into five size groups. By ap-

plying the polyacrylamide gel electrophoresis the same authors were able to resolve AHS virus into six definite segments and finally basing on the thermal denaturation curve and the resistence to RNase hydrolysis, they suggested that the virus has a double-stranded nature.

However, since AHS purified virus was shown to possess isometric capsid with icosahedral symmetry and pseudo-memrane of host origin, resisted lipid solvants, had a double-stranded RNA and other characteristics of Reovirus, it was provisory classified, by the International Committee on Nomenclature of Viruses, as a possible member of Reoviruses.(95)

Storage and thermal stability.

The effect of temperature on viability of AHS virus has been studied by several workers. M'Fadyean 1901 (40) reported that infective citrated blood plasma was not inactivated after heating for 10 minutes at a temperature of 55°C. Similar material after 10 minutes at 75° C. still produced horsesickness on injection into a susceptible horse. Theiler 1930(93) has shown that the virus in a mixture of equal parts of infective blood and Edington's preservative, remained viable for 4 years, and that it was destroyed at 37° C. and 45° C. for 14 and 6 days, respectively.

Alexander 1935 (5), studying the effect of various temperatures in keeping qualities of AHS virus, stated that the titer of a neurotropic virus suspended in a 10% serum-saline and kept at 4°C, did not decreased remarkably after 6 months. The virus was gradually destroyed at higher temperatures and the thermal death range was 55-60° C.

In cell culture, the virus, in the medium containing calf serum, was found to be stable at 4°C. for 3 months, but at -25°C. a decrease of 4 log. in titer was occured within 48 hours unless the virus was diluted in a stabilizer containing lactose 5%, peptone 1%, in the phosphate buffer M/50, PH 7.2-7.4 (Ozawa et al.1965) (63)

At 25°C. a fall of 2 log. has been observed, after 40 days, in the titer of virus stabilized with 5% calf serum (Mirchamsy and Taslimi 1967) (52). At 37°C. in absence of serum a decrease of 2.5 log. in titer was noticed, but when 1% of calf serum was added the fall of titer in the same period of time was 0.8 log. (Hazrati and Ozawa 1965) (26). The same authors (63) have observed a decrease of 1.7 log. in titer of virus stabilized with a solution of lactose-peptone and kept at 37°C. for 24 days. At the same temperature after 40 days Mirchamsy and Taslimi (52) have recorded a fall of 6 log. in titer of AHS virus in cell culture medium supplemented with 5% of calf serum.

At –40°C. a decrease of 2 to 3 logs. in 4 months has been observed. This was prevented by adding 1% gelatine or 5% lactose, (Mirchamsy and Taslimi 1964) (44).

Inactivation of AHS virus, grown in tissue cultures, at –20°C. and –30°C. was attributed to the presence of salts such as NaCl, CaC12, and MgC12, contained in the culture medium, by Ozawa and Bahrami. (73) They found that while the infectivity of neurotropic and viscerotropic AHS virus, suspended either in tissue culture medium or phosphate buffer saline, decreased markedly at –20°C. and –30°C., the titer would be rather stable in the solutions without these salts. The same authors stated that the infectivity of AHS virus in these temperatures was protected when lactose, sucrose or glucose was added to the virus suspension at the rate of approximately 5%,and that the virus at –70°C. even in presence of salts was stable.

Rapid dessication in the frozen state or at room temperature has no ill effect on the virus. Lyophilisation seems to be the best means to keep the virus viability (Howell 1964) (33). A lyophilized vaccine kept at 4°C. lost only 1.5 log. of its titer after 18 months. (52)

The pH of the virus suspension plays an important part in conservation of AHS virus. A cell culture neurotropic virus kept at pH 6.2 at 4°C. lost 2 log. of its titer in 3 hours, while titer of the same virus with a pH value of 6.5 to 8.0, kept at the same temperature, did not change considerably after 6 days (Ozawa et al 1965) (63).

pH Stability.

A pH value of 5, was found to inactivate the virus completely within 5 minutes. (5) A remarkable decrease was also observed in the infectivity titer of a virus suspension with a pH of 6.2, within 3 hours storage at 4°C. (63)

The virus, however, resists well the pH changes on the alkaline side of neutrality up to pH 10, the optimum pH value being 7 to 8.5.

Resistence to lights.

Alexander (1935) showed that the infectivity of an infective mouse brain suspension was not affected by exposure of the suspension to a bright diffused light of the laboratory for several weeks. He found that although irradiation with an artificial light was not virucidal, its combination with methylene blue had a marked inactivation effect on AHS virus. He also indicated that the virus was protected from the photodynamic action of the methylene blue by the

presence of living cellular elements or fragment of them in the virus suspension.(5)

The susceptibility of AHS virus to ultra violet light was first reported by Alexander, who found that light, from a 220 volts 3.7 amp. Hanau quartz mercury lamp, from a distance of 30 cms., completely inactivated the virus, in a fluid with 4mm. depth, within 1 minute.(5) It was demonstrated, later on, that this susceptibility was depended on the antigenic types of AHS virus, and that the rate of inactivation of heterologous strains of the virus was so markedly different that it could be used as a means for differentiating antigenically different strains of AHS virus. (78)

Effect of chemical agents.

Ethyl ether at a concentration of 2 per cent mixed with the virus suspension has no ill effect on the virus after a contact of 1 month at 30°C. (Alexander 1935) (5), the virus was not also significantly inactivated at 4°C. for 18 hours in the presence of 20 percent diethyl ether (Howell, 1962). (32)

Sodium desoxycholate at a final concentration of 1/1000 has no effect on the virus but saponin, normally used to enhance the immunity responses, inactivates it. (86)

Glycerin is used for conservation of the virus in fluid state (5). Edington's, oxalate–carbolic acid–glycerin (0.C.G.), diluent, normally used as an anti-coagulant and preservative, (92) has no ill effect on the virulence of the virus.

Heparin, was shown to have an inhibitory effect on viral synthesis in cell culture (Mrichamsy and Taslimi, 1965). (46)

Neutral glycerin at 50% in distilled water is a suitable preservative when pieces of spleen or other organs are to be preserved for a short time.

AHS virus was found inactivated when formalin, at a concentration of 1/3000 was added to the virus suspension and the mixture was kept for 5 days at 4°C. (Ozawa and Bahrami, 1966) (66). Mirchamsy and Taslimi, 1968 (53), were able to inactivate AHS virus by shaking a virus suspension for 48 hours in a water bath of 25°C. in the presence of 1/8000 formaldehyde. The same authors successfully inactivated AHS virus with 0.2 per cent of ß – propiolactone after 15 minutes incubation in a 36°C. water bath. (53)

Biological and immunological properties.

Antigenic plurality and virulence variation.

The antigenic heterogenecity of different strains of AHS virus has long been known by South African scientists. Theiler (87,88,92) was the first who observed immunized horses, perfectly resisted challenge with homologous virus,

sometimes contracted the disease when challenged by heterologous strains. He also observed that AHS was relapsing among immunized equidae because of the antigenic variation of the virus. The same author found that among homologous strains, there was a wide range of virulence for susceptible equine. Theiler was finally able to find some antigenic relationship among different strains of the virus and accordingly he first established two immunizing strains for horse and one for mule to produce a fairly successful vaccine with his serum–virus method of immunization.

Despite the apparent success achieved by Theiler, the problem of antigenic plurality was not solved until AHS virus was successfully adapted into the brain of adult mice (2,4,55,56). The introduction of mice as a susceptible host to AHS virus facilitated the technique of neutralization test. The original technique developed by Alexander was based on intracerebral injection of virus–serum mixture in mice, and was proved to be useful for immunologic studies of the virus. By this economical and reliable method, Alexander was able to study a large number of strains isolated during several years and to select antigenically different strains for incorporation in a polyvalent vaccine.

Although the introduction of intracerbral neutralization test was a great step to solve the problem of identification of antigenically different virus strains, but as the horse convalescent or hyperimmune serum was used for neutralization test, the frequent presence of heterologous antibodies made the interpretation of experimental results very difficult. To overcome this problem, McIntosh (39), using the same method and type specific antisera prepared in hyperimmunized rabbits, classified 42 strains of the virus, isolated in Africa, into seven distinct immunological types. Subsequently two additional new types of AHS virus, namely types 8 and 9, were identified by Howell, (32) and the existent types of AHS virus was increased to 9 distinct antigenic types.

In assessing the antigenic type plurality of AHS virus, one may ask the origin of the types and the mechanism of the possible mutation that may occur in reservoirs or in vectors in interepizootic periods. These questions will not be answered at the present time with the lack of knowledge about the reservoirs of the virus and the real cyclical developement of the virus in the specific vectors.

Another point of intrest is the wide range of virulence of the isolated virus strains in nature (92). Experimentally, virus strains adapted in mice lose their virulence for susceptible equine slowly, while in cell cultures, the fall of virulence for horse is very fast. Mirchamsy and Taslimi (44), have observed that after 8 to 13 passages of an AHS virus strain in MS or in BHK cell lines, the virulent virus lost its viscerotropic nature. After 23 passages, the infectivity, of the same virus strain, for mice decreased considerably and it was also

avirulent for equidae producing a low antibody titer when it was used for immunizing foals. According to these authors there was some correlation between the neurovirulence of AHS virus for mice and its antigenicity for horses. In another report, Mirchamsy and Taslimi (50) showed that an AHS virus strain avirulent for mice was unable to produce immunity in horses. They have developed two avirulent AHS virus strains for mice by serial passages in MS cells at decreasing temperature. These two mouse avirulent strains were unable to induce detectable antibody in horses. Another characteristic of these avirulent strains was their potential ability to produce interferon more than the parent virulent virus strains.

Adaptation and multiplication of the virus in animals.

(Solipeds).

Horses are the most susceptible species to the virus of AHS both under natural and experimental conditions. In nature, the disease is transmitted to healthy individuals, mechanically or biologically, by the insect vectors previously fed on the infected animals during the viraemia stage.

Under experimental conditions, horses became readily infected by intradermal, subcutaneous, intratracheal, intrapulmonar, intramuscular, and intravenous routes. The infection may be produced by ingestion of infective materials only when a large doses was administrated. Intravenous inoculation, however, was found the most reliable route of infection. Fully susceptible horses may become infected by as small dose as 0.0001 c.c. blood of a virulent strain. The reaction of the horse, differs, both in severity and the length of incubation period, according to the virulence of the virus strain, as it was shown that there existed a variation among natural strains in their virulence for horses.

In reacting horses, naturally or experimentally infected, the virus is present in blood during the febrile stage, and, at a lower concentration, in internal organs. Spleen was found to contain virus in high titer and for the same reason was the tissue of choice in the preparation of formalized tissue vaccine. Splenic tissue, as well as, blood collected at the height of thermal reaction could be kept, under favourable conditions, for a long period of time and be used as the source of virulent virus for control the innocuity and efficacity of the vaccines.

Horses recovered from infection developed antibodies which could be used in several serological tests.

Mules, being less susceptible, could be equally used for the same purposes. Donkeys and wild soliped animals, on the other hand, were found to be more resistent to the virus and have not been used in experimental works.

(Mice).

Mice were first reported to be susceptible to AHS virus by Nieschulz (1932, 1933) and Alexander (1933), who, independently and almost concurently, succeeded in transmitting the viscerotropic AHS virus, by the intracerebral route, to Swiss albino mice. (2,55,56)

Subsequently, it was reported that by subcutaneous or intramuscular inoculation of the virus it was not possible to infect mice, and that by intraperitoneal route, infection may be established only when massive doses of certain strains were administrated.(4)

Kulenkampf, on the other hand, infected 66.9 to 100 percent of mice by intransal instillation of mouse neurotropic AHS virus. (36) This was confirmed in a recent publication by Ozawa and Dardiri (1970). They reported that from all possible routes of contact infection, i.e. oral, conjunctival, intrarectal, and intranasal routes, mice were infected only by intranasal inoculation. All the mouse adapted neurotropic strains of AHS virus, except those belonging to type 8;and viscerotropic strains of at least type six and nine could readily infect mice when were administrated by intranasal route. They concluded that this way of direct contact infection could occur in nature when brain of infected mice is eaten by healthy ones, during which, an intranasal instillation may happens. (75)

In spite of these findings, the risk of accidental infection, by direct or indirect contact, in mice is in the minimum and still mice should be considered as an exceedingly suitable laboratory animal for isolation and identification, neurotropic fixation, attenuation and other studies on different properties of AHS virus.

Young mice of approximately two months old was first strongly recommended for these purposes (4). The animal when injected intracerebrally become easily infected. In the first two passages the mortality rate is not very high and only some of the inoculated mice, approximately 20 per cent, become infected. The incubation period varies between 9 and 26 days (83). As the serial passages progress, the viscerotropic virus undergoes a metamorphosis and becomes adapted and accustomed to neurotropic propagation, the incubation period shortens gradually and mortality is usually 100 percent and the interval between injection and death reaches a constant minimum of 3 to 5 days.

Adaptation and neurotropic fixation of AHS virus by intracerbral passages in mice, however, contain some peculiar features which could be summarized as follows:

The virus multiplies only in nervous tissues, and when injected intracerebrally, may be detectable in the brain before the appearance of any clinical symptoms in inoculated mice. In brain, virus obtained its highest concentration just before death.

Suckling mice of 2 to 4 days old proved to be more susceptible to AHS virus by intracerebral injection (25,37). The infection by intraperitoneal inoculation and intranasal instillation of the virus was also reported (21). The incubation period in suckling mice, inoculated intracerebrally by the infected horse blood, varies between 4 and 20 days, which reaches a minimum of 2 to 4 days after 2 or 3 successive passages. (25,37,83) The mortality rate may reaches 100 per cent from the first generation. This high susceptibility, together with the fact that virus yield per unit weight of suckling mouse brain was much higher than in adult mice (21), made suckling mice the animal of choice for isolation of the virus from infected equine, as well as, for preparation of various AHS antigens.

Alexander (1933) noticed that the increase in virulence of a strain of AHS virus for the mouse, during intracerebral serial passages, was accompanied with a decrease of its virulence for the horse(2). It was, subsequently, found that through successive intracerebral passages in mice, all viscerotropic strains of AHS virus become attenuated while retaining their antigenic and immunizing properties. (3,4) The representative strains of different established immunological types, being attenuated by approximately 100 intracerebral passages in adult mice, have been employed for preparation of "horsesickness neurotropic mouse brain vaccine" and "horsesickness neurotropic tissue culture vaccine" which have been extensively used within last 40 years. (39,63)

In attenuation of AHS virus strains in adult mice, the number of intracerebral passages is very critical. It was found that the virus strains did not become attenuated at the same rate. Certain strains after variable number of intracerebral passages in adult mice still produced very severe reaction in fully susceptible horses, while another strain at the same passage level, may become so attenuated that, in spite of even repeated vaccination, failed to provide complete protection against natural exposure (3,21)

The neurotropic virus strains, at various passage levels, are pathogen for different breed of mice, rats, a species of gerbille, guinea pigs, (4) hamster, (85), and lambs, (35), by intracerebral inoculation. The high tropism of neurotropic strains of AHS virus to multiply in the brain of animals other than mice, rose this question whether neurotropic strains had any affinity to nervous tissues of horse, from the time of introduction of neurotropic vaccine.

Alexander, however, showed that horses injected intracerebrally with various passage levels of one selected strain did not produce a fatal encephalitis and concluded that the neurotropic vaccine being inoculated subcutaneously would be perfectly safe.(6) This became a general belief and even cases of blindness in mules and madness in horses, which occured here and there after vaccination, had not been attributed to the neurotropic affinity of the neurotropic vaccine strains for these animals.(7)

However, when encephalitis with fatal results among fully susceptible equine, following vaccination with polyvalent mouse neurotropic vaccine, were reported from several countries (34,61,82), the possibility of neurotropic affinity of the vaccine strains received more attention. Isolation of type 2 AHS virus from the brain of vaccinated equine in Israel and India, (59,76) as well as, the isolation of types 1 and 2 neurotropic vaccine strains from two different donkeys in Iran, (72) indicated that these strains may multiply in the brain tissues of equine producing blindness, neurologic disorders, and fatal encephalitis in vaccinated animals. Erasmus studying the neurotropic characters of vaccine strains in guinea pigs and horses, confirmed this and demonstrated that all virus strains attenuated in mice were potentially neurotropic for horses, and that the nervous disorders could only be produced in fully susceptible animals during primary vaccination. (18,21).

Suckling mice could be used for neurotropic fixation and attenuation of AHS virus. It was found that the virus will be attenuated more readily in this host, developing lower degree of neurotropism, as compared with adult mice, and thus could be used for immunization of equine with more confidence. (21)

(Guinea pigs)

The susceptibility of guinea pigs to AHS virus was first reported by Alexander, and then was confirmed by Nieschulz, almost immediately after adaptation and neurotropic fixation of the virus to the mouse. (2,56) No data of any attempt on fixation of a viscerotropic strain of AHS virus in guinea pigs is available, but according to Nieschulz, intracerebral inoculation of this animal by infective blood from a horse resulted to no demonstrable reaction. AHS virus, however, after being passaged for a few generations in mice, will infect guinea pigs very easily.

The infection in guinea pigs is characterized by a short incubation period, appearance of a diphasic febrile reaction, development of nervous symptoms which is mostly accompanied by sudden drop of body temperature, and death.

Alexander(4), stated that direct intracerebral route of inoculation was the most certain method in infecting guinea pigs with AHS virus. Virus admi-

nistrated in this way multiplied in the brain and spread centrifugally in the nervous tissues. The virus obtained its highest concentration in brain, just before death, and this was usually 10 to 100 times lower than the concentration of the virus in mouse brain.

The same author indicated that once the virus become adapted to guinea pigs, further passages progressed with no difficulty, and that by intracerebral passages a progressive increase in virulence of the virus for guinea pigs was observed. Neurotropic fixation was also accompanied with the attenuation of the virus for horse. The attenuation rate seemed to be more rapid through guinea pigs than through mice.

Guinea pig neurotropic strains of AHS virus were experimentally used for immunization of horses and mules (3), but in spite of satisfactory results, due to greater ease of propagation of the virus in mice, guinea pig neurotropic AHS virus has never been used, in a large scale, for immunization of equine in the field.

Guinea pigs were found to be also susceptible to neurotropic strains of AHS virus by routes other than intracerebral inoculation(4). Alexander demonstrated that 60 per cent of guinea pigs inoculated intraperitoneally with a selected neurotropic strain developed a fatal infection, and that some of the recovered animals found to be immune to a further infection. Erasmus in 1963, studying the neurotropic affinity of mouse attenuated vaccine strains for guinea pigs, found that type 7 (Karen strain) was markedly neurotropic for guinea pigs on intraperitoneal injection. He, subsequently, using the same strain, showed that guinea pigs inoculated by intramuscular, subcutaneous, intranasal, and intrarectal routes, died of a viral encephalitis, while thos infected by oral and conjunctival routes showed no reaction. When guinea pigs were inoculated intraperi toneally, by neurotropic strains of other types of the virus, reacted quite differently. Certain strains produced a febrile reaction with a lower mortaliy rate, and some developed no detectable clinical reaction. (18)

Exposure of guinea pigs to strains of different types of AHS virus by intranasal instillation, on the other hand, resulted to a marked febrile reaction, which terminated to a viral encephalitis and death, in majority of the subjects. This finding indicated that exposure of guinea pigs to a certain neurotropic strain of AHS virus by intranasal route was the most sensitive method of demonstrating relative degree of neurotropism of the virus (18), and that guinea pig, apart from its use for preparation of specific HS antiserum, could be considered as a valuable laboratory animal for determining the innocuity of mouse neurotropic attenuated AHS virus strains.

(Ferrets)

McIntosh, in 1953, reported the susceptibility of ferrets to the viscerotropic strains of AHS virus by intraperitoneal and intracardial inoculations, and presented a relatively reliable means for isolation of virus especially from horses whose immunity has been broken down during a natural infection. He, after several unsuccessful attempts to isolate AHS virus from the blood of infected, previously immunized, horses by the usual intracerebral inoculation of suckling mice, was succeeded to infect ferrets by intracardial inoculation of the same infective materials. In this experiment ferrets simoultaneously inoculated intraperitoneally showed no apparent reaction and isolation of virus from them failed. The virus was isolated from the blood and spleen of the infected ferrets, during the febrile reaction in suckling mice. (37)

Reports have not been found on infection of ferrets with AHS virus by any other routes of inoculation.

(Hamsters)

Sharma, in 1968, succeeded to adapt a mouse neurotropic strain of AHS virus to young adult golden hamster by intracerebral route. The infection was very similar to that in mice and guinea pigs. The incubation period, being longer than that in mice, was quite irregular in the first few passages, then became regular varying between 6 and 9 days.

The concentration of virus in the brain of infected hamster appeared to be approximately equal to that in mouse brain. (85) This high infectivity titers promises the hamster to be of potential value as a source of horsesickness antigens for various serological tests, whenever, the use of infected mouse brain is not advisable.

No effort has so far been made to adapt the viscerotropic strains of AHS virus to hamster, nor the other routes of infection have been looked for.

(Rats)

Alexander demonstrated that when different strains of rats (wild brown rats and albino rats) were injected intracerebrally by a mouse neurotropic strain of AHS virus, became infected and some died. The virus was multiplied in brain and retained in rats by serial intracerebral passages. Within 16 passages, an increase in virulence of the virus strain for rat was observed, but the concentration of virus per unit weight of brain tissues always remained unexpectedly low, approximately 1000 times lower than that in mice.(4)

Rat, however, has not been proved as being a suitable laboratory animal for research purposes on AHS and thus no effort was made to determine its susceptibility to viscerotropic strains of the virus, or the possibility of its infection by the other routes.

(Other rodents)

The multimammate mouse (Mastomys coucha), and young gerbille (Tatera lobengula) were found to be fully susceptible to the intracerebral injection of mouse neurotropic strains of AHS virus. The course of the disease in multimammate mouse was 1–2 days longer than that in common white mouse. The disease in gerbille showed a clinical course similar to that observed in rat(4).

(Dogs)

In 1907, Theiler, by intravenous inoculation of dogs with blood from horse with AHS, showed the susceptibility of this species to AHS and indicated that the disease in dog has a very rapid course both in incubation period and thermal reactions. (89).

M'Fadyean, on the other hand, following a series of experiments gave results contrary to those obtained by Theiler, and reported that from 9 dogs inoculated subcutaneously with infected blood none became visibily ill. He stated that, by injection of susceptible horses with blood samples collected, on fourteen days post inoculation, from 2 of these dogs no obvious effect which could be attributed to presence of AHS virus in dogs blood was observed. (41)

Theiler, in 1910, reporting the results of further experiments, re-affirmed his first view and showed that dogs inoculated subcutaneously, even with diluted and filtered horsesickness infected blood, reacted as those inoculated intravenously in his previous experiment, and some died with pulmonary form of the disease. He indicated clearly that the blood of reacting dogs at the height of the febrile reaction, were able to produce fatal acute AHS when inoculated into susceptible horses and mules. Furthermore, he stated that the viscerotropic virus of AHS presented in the blood of an experimentally infected dog during the short febrile reaction, was successfully transferred from dog to dog, and that during passaging the virus retained its viscerotropic nature, so that the virus at different passage levels, including the last generation (the thirtieth) was still fatal for horses. In the course of this experiments 91 dogs were used of which 24 died of horsesickness (26.4%), 53 showed reactions and recovered (58.3%), and 14 showed no reaction whatever (15.3%). (91)

Outbreak of AHS among dogs have been reported on only three occasions. (13,24,77) In each occasion dogs, after being fed with raw meat of horses which had apparently died from AHS, became sick, and showed symptoms of horsesickness which varied considerably in severity. The fact that horsesickness was indeed widespread among the dogs was confirmed, in one occasion, by reproducing the disease in horse by inoculationg with the blood of the sick dog, (13) and in the other, both by isolation of the virus from a fatal case and by positive serum neutralization test in the majority of dogs recovered from infection. (77)

In a recent study on susceptibility of dogs to neurotropic and viscerotropic AHS virus, it was reported that exposure of dogs to neurotropic type 2 of the virus by oral, nasal, ocular, intracerebral, and intravenous routes, produced CF and virus neutralizing (VN) antibodies. A marked VN antibody was also observed when dogs were inoculated intravenously with viscerotropic, type 9, virus. The same viscerotropic virus strain on the other hand did not develop a serological response in dogs by oral route unless the dogs were fed with adequate amount of infected blood and meat. (14)

In spite of all the evidence indicating that dogs can be infected artificially or by ingestion of infected meat, McIntosh doubted whether this animal are ever infected, under natural circumstances, by insect-borne AHS virus. He found that, in an enzootic horsesickness area, only 1 out of 13 dogs had horsesickness specific neutralizing antibody in its serum and concluded that the vector which infect the horse does not readily feed upon, or while feeding upon the dogs, does not readily infect this species, and therefore it is improbable that canine species plays an important part as a reservoir in the spread of horsesickness.(38)

(Rabbits)

Alexander recorded his inability to infect rabbits with mouse and guinea pig neurotropic virus and concluded that rabbit is insusceptible to AHS virus.(4) This was confirmed by several other workers, who indicated that although rabbits could not be infected with virus of AHS, by intravenous, intraperitoneal, intramuscular, and subcutaneous inoculation, but reacted serologically and were found to be the laboratory animal of choice on producing AHS hyperimmune serum for various serologic tests. (26,28,39,45)

(Chickens)

Chickens, while being fully resistant to neurotropic AHS virus, were found to be a good producer of precipitating serum to horsesickness virus, although some individual differences were observed. (28)

(Other animals)

In spite of few reports on transmission of AHS to cattle and sheep (17,94), these animals have not been found to become readily infected (90). However, in a recent publication, it was stated that a mouse neurotropic virus strain produced a fatal disease, when inoculated intracerebrally, in lamb. The incubation period of the disease was 15 to 30 days which decreased to few days after several serial passages in lamb. (35)

It was also demonstrated that sheep hyperimmunized by a series of in-

travenous inoculations of AHS virus, produced specific neutralizing antiserum. (26)

Following observation on susceptibility of goats to AHS (17), Theiler stated that a mild transient febrile reaction could be produced in Angora goats after intravenous inoculation with viscerotropic strain of AHS virus. He also reported that not all of inoculated goats reacted in his experiments.

Theiler succeeded in reisolating the virus from reacting goats, and showed that blood of these animals was infective for other goats and for dog but not for horse. In a later publication similar observations were reported in goats of West Africa, infected in the same way. (15)

Pigs, cats, and monkeys are not susceptible to AHS virus. No report on susceptibility of human being is also available.

Adaptation and propagation of the virus in chicken embryos.

Alexander (1938) (9) was the first who cultivated a neurotropic strain of AHS virus in chicken embryos by inoculating on the chorioallantoic membrane. The maximum yield of the virus was obtained 4 to 5 days after inoculation. He was unable to adapt a viscerotropic virus strain in this host system.

McIntosh, as mentioned by Howell, (33) was able to propagate two viscerotropic strains of AHS virus in yolk sac of 8 or 9 days old developping chicken embryos at 32°C. The first few passages of the virus in yolk sac were highly pathogenic for baby mice but the virus lost, gradually, its pathogeny for adult mice when the number of passages increased.

Egg adapted virus at various passage levels proved to become attenuated without loosing of antigenicity for horse. This may be taken as an evidence of giving chicken embryos a potential value for vaccine production, but the experiments of McIntosh, due to low concentration of the virus, and the original work carried out by Alexander were unsatisfactory.

Goldsmit (23), however, recently developed six neurotropic and one viscerotropic strains of AHS virus in 8 days old chicken embryos by the yolk sac route of inoculation. The titers of virus strains in infected embryos, although varying according to the strain, for some strains were high and comparable with those obtained in mouse brain. The neurotropic characters of the neurotropic strains retained after six consecutive passges in fertile eggs.

Adaptation and propagation of the virus in cell cultures.

AHS virus was first adapted to primary hamster kidney cells by Mirchamsy and Taslimi (1962, 1963) (42,43). Characteristic cytopathic changes were observed and the occurrence of an eclipse phase of 8 hours, followed by an increase of released virus, was noticed. The virus was then passed to baby hamster kidney cells and to hamster kidney cell line, BHK 21. (44) The yield of virus in baby hamster kidney cells was at least 1 log higher than that of adult hamster kidney cells. Virus titer, after 4 and 6 serial passages in baby hamster kidney cells, of a wild strain type 9 of AHS virus were 7.97 and 7.94 log 10, respectively.

In hamster kidney cells infected by a virulent strain of AHS virus type 9, the first cellular changes were observed 36 hours after infection, in the second passage of the virus. Infected cells lost their characteristic shape and became rounded and distorted. The change increased on the third day when in all parts of infected sheet a marked cytopathological effect (CPE) was noticed. At this stage the cytoplasm was granular and contracted. The nucleus was pyknotic and disintegrated. This progressive degeneration was completed on the fourth day when the infected cell sheet was practically detached from the glass surface.

The virus was adapted, by Ozawa and Hazrati (1964, 1967) (26,71), to monkey kidney stable (MS) cell line, BHK 21, and to vero cell line. The MS cells have been extensively used for serological studies of AHS virus and for live and killed AHS vaccine preparation. Ozawa and hazrati recorded the first CPE in MS cell cultures at the first passage of most strains of AHS virus studied. The characteristic CPE was rounding of the infected cells. No cytoplasmic inclusion bodies was found in the infected cells. The nuclei of the infected cells were, however, darkly stained with Giemsa stain. After 5 to 7 passages in MS cells, complete CPE were observed on 3rd day after infection.

In coverslip cultures stained with hematoxylin-eosin or by Feulgen's method, the 1st changes were visible 16 hours after infection. The nuclei became larger and there was an increase in Feulgen-positive substance, basophilic granules which filled the enlarged nuclei, and the nuclei were eosinophilic. 24 hours after infection, accumulation of basophilic Feulgen-positive substance in and around the nuclei were noticed. At 31 hours after infection, floculation of the rest of basophilic substance became evident, and most of the aggregate was attached to the nuclear membrane. Between 31 and 40 hours after infection, the basophilic aggregates attached to the nuclear membrane increased in size and eventually formed a few large basophilic Feulgen positive bodies. Between 40 and 48 hours after infection, nuclei became pyknotic and clearing of nucleoplasm was observed. The Feulgen positive bodies with smooth surfaces were seen in the clear glassy or empty nuclear background.

Chicken embryo fibroblast cells were also found to support the growth of low-passage mouse brain adapted virus (Erasmus, 1963) (19). No specific CPE was, however, observed.

The virus was also adapted, by Erasmus (20), to calf kidney, horse kidney, and lamb kidney cell cultures.

More recently Mirchamsy et al. (54) adapted two strains of AHS virus, previously adapted to mouse brain or to mouse brain and MS cells, in a mosquito (Aedes albopictus) cell line. The peak titer of cell released and cell-associated virus were observed 96 to 120 hours after cells were infected. In the growth curve studies, the cell associated viral titer was nearly the same as or slightly higher than that of cell released virus. CPE were not observed in infected mosquito cells, but viral antigen was detected in cytoplasm and around the nucleus of infected cells by fluorescent antibody technique and by acridine orange staining.

In a search for visualization of AHS virus by fluorescent antibody technique in MS cells and by acridine orange staining, Mirchamsy and Taslimi, (45,47) have detected the first antigen, 8 hours after infection, around the nucleus and later on large amount of granular of diffused antigens were found in cytoplasm of infected cells. These authors believed that the nucleus may play a role in synthesis of the new virion which matured in cytoplasm. This finding was confirmed by Ozawa et al (1966) (70) who have used the same cell system for growth of AHS virus. In this study, accumulation of Feulgen-positive substance about the nucleoli during the course of infection, and oval dense bodies of 45 to 75 mμ. in diameter, inside the infected nuclei and cytoplasm at a later stage of infection was observed, and it was stated that AHS virus was reproduced in the nuclei, and that the dense bodies, observed in cytoplasm, possibly represented denaturated cytoplasmic particles.

In another immunofluorescent studies, in MS and VERO cells, Ozawa (74) has indicated that the first fluorescent antigen was detected in cytoplasm of both MS and VERO cells. It was also noted that in both cells a large proportion of infected cells showed spherical fluorescent bodies in or on the top of the nuclei.

The in vitro quantitation of different types of AHS virus by plaquing the virus in MS cells under agar or other overlays have been independly studied by Hopkins et al (1966) and by Mirchamsy and Taslimi (1966) (30,48). Under agar overlay, large and small plaques were observed. The purification of large plaques by cloning was possible. By addition of protamine sulphate to the agar overlay only large plaque were produced. The plaque size variation could not be used as a suitable marker for determination of variants of AHS virus.

Maintenance and transmission of the virus in nature.

Transmission of AHS virus by various species of arthropods was suspected, without adequate experimental evidence, long before 1944, when Du-Toit (16) produced the disease by inoculating horses with a suspension of culicoides caught in the field. Since the appearance of this report, culicoids spp. have been known as essential vectors of AHS and the virus was accordingly classified by Andrewes (10) as a culicoides-borne arbovirus.

Mosquitoes, as possible vectors of AHS virus, were studied by several workers. Nieschulz et al, (57), and Nieschulz and DuToit (58), however, indicated that mosquitoes of the genus Aedes, although they harbored the virus for one week after experimental feeding, could not transmit the disease. Ozawa et al· (65,68), transmitted AHS by means of the bite of Anopheles stephensis and Culex pipiens which had engorged infected horse blood 15 to 22 days previously, and by the bite of Aedes aegypti which had been fed with viral suspension. Results of further investigations (67), indicated that the virus remained in Aedes aegypti for more than 5 weeks.

In order to provide further evidence of the possible contribution of mosquitoes in natural transmission of the disease, Mirchamsy et al., experienced the growth of AHS virus in mosquito cells. They adapted two strains of type 9 AHS virus, previously adapted to mouse brain or to mouse brain and in monkey cell line (MS), in a mosquito (Aedes albopictus) cell line. (54) It was found that propagation of AHS virus in this host system was not followed by cytophatic changes by maturation of virion in cytoplasm and persistent infection of the cell line were shown by fluorescent antibody technique and by acridine orange staining. Subsequently it was shown that this mosquito cell line became a chronic carrier of AHS virus and even after 15 subcultures of cells the titer of virus was not significantly decreased (Mirchamsy et al. Unpublished data).

Despite the success achieved in propagation of AHS virus in a mosquito cells, which in turn is in favour of possible biological transmission of AHS virus by mosquitos spp., it must be remembered that in this study, as well as, in most other transmission experiments so far published by various workers, the lack of evidence for biological transmission of the virus by a given vector is quite possible. As a matter of fact, AHS virus was shown to be highly resistant to heat, so that, for example, a suspension of virus could be kept for 40 days at 25°C. without great loss of its titer. (52)

AHS virus, is transmitted not only by insect vectors biologically, but also it could be transferred, mechanically, from infected individual to highly susceptible equine population, by nocturnal biting insects. Several biting insects such as Anopheles spp., Stomoxys calcitrans, Lyperosia minuta, different dip-

tera, some species of culicinea, and ticks have been incriminated to be the mechanical viral transmitters. (82)

The key point in transmission of AHS virus is to know the reservoir hosts which maintain the virus during the winter and in period of interepizootics. According to the previous experiences, domestic equidae, dogs, or culicoides spp., could not be considered as reservoir hosts. Horses recovered from the disease can not kept virus for a long time in their bodies and the amount of the virus in recovered horses, having a high titer of neutralizing antibody when it existed is so low and so reachless that the transmission of the disease by recovered solipeds must be excluded.

In a search for the reservoir hosts among wild animals, Theiler carried out extensive transmission experiments from a variety of wild mammals, birds, and amphibians, that were caught or shot in enzootic area, all with negative results. Recently a large number of sera from African wild games were subjected to serological investigations. It is intresting to note that a large number of elephant and zebra sera showed positive titers of neutralizing antibodies against different serotypes of AHS virus (Hazrati, unpublished data). This finding may has an important value for better understanding the fate of AHS virus in interepizootic periods, if sera from elephants and zebra from area free ofAHS could be obtained for similar serological studies.

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