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Experimental Infection of Chimpanzees with Ťahyňa Virus by *Culiseta annulata* Mosquitoes

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Abstract. The transmission and course of the Ťahyňa virus infection induced by the infected *Culiseta annulata* mosquitoes in chimpanzees are described in this paper. Four out of five chimpanzees exposed to infected mosquitoes became sick. The course of the Ťahyňa virus infection in chimpanzees was characterised by the rise of body temperature, viremia and the virus neutralizing, hemagglutinin inhibiting and complement fixing antibody formation. In two chimpanzees also the acceleration of erythrocyte sedimentation was observed. Infection could be caused even by three infected mosquitoes feeding on the animal. The usefullness of chimpanzees in studying the acute human Ťahyňa virus infection is discussed.

The high frequency of Ťahyňa virus antibodies in human population and the serologic results providing the evidence of acute human infection with the Ťahyňa virus on the one hand and the lack of adequate laboratory evidence of clinical illness caused by the Ťahyňa virus on the other, led us to undertake the experimental Ťahyňa virus infection in chimpanzees which were found suitable for studies with this virus (ŠIMKOVÁ, BÁRDOŠ 1966). To simulate as closely as possible the conditions occurring in nature we used mosquitoes for inducing the infection and we worked with an extraneural variant of the Ťahyňa virus at a low passage level not inoculated intracerebrally during passaging. We carried out the experiments with the mosquito species *Culiseta annulata* (Schrk.) in which the overwintering of Ťahyňa virus was proved experimentally during its hibernation (DANIELOVÁ, MINÁŘ 1969).

MATERIAL AND METHODS

Experimental animals. Young chimpanzees (*Pan troglodytes*), females and males ranging from 4.1 to 4.7 kilograms in weight, were quarantined singly or in pairs in the cages during five weeks prior to experiments. At this time the health condition of each chimpanzee was checked by systematic physical examinations, hematological and serological tests. The animals were fed on the usual diet of monkey-food supplemented with fresh vegetables.

The mosquitoes of the *C. annulata* species were collected as imagoes in the caves near Beroun (central Bohemia). They were kept at the temperature of 25—26 °C and 90 % relative humidity. They were fed with a 10 % glucose solution.

Virus and infection of mosquitoes. The "236" strain of Ťahyňa virus isolated from mosquitoes by intramuscular inoculation of Syrian hamsters (BÁRDOŠ, DANIELOVÁ 1959) was used in its 10th extraneural passage.

The mosquitoes were infected by feeding on viremic hamster blood to which 5 % of glucose had been added. The blood meal was exposed to the mosquitoes for three hours. After finishing the exposure, the blood-glucose solution was titrated by intracerebral inoculation to 8 g white mice.

Exposure of chimpanzees to mosquitoes. The chimpanzees were anesthetized with pentobarbital (Thiopental) injected subcutaneously (30 to 40 mg/kg of body weight) in the nuchal region of animals. For engorgement on the chimpanzees the mosquitoes were confined in small silon cages which were fixed at the shaved skin on the ventral thoracoabdominal region of the sleeping chimpanzees, so that the mosquitoes could feed without difficulty (Fig. 1). Various numbers of mosquitoes were allowed to feed on each chimpanzee. The cages with mosquitoes were applied gradually to chimpanzees so that each animal was exposed to the mosquitoes for three to three and a half hours. Subsequently to the exposure the mosquito infection rate by the isolation experiments from the individual engorged mosquitoes and average virus levels both in the engorged and unengorged mosquitoes were established.

Fig. 1. Exposure of chimpanzee to mosquitoes.

In the isolation experiment 1 mosquito was ground in 1 ml of the 10 % guinea pig serum in pH 7.4 saline with 1000 units of penicillin and 1000 γ streptomycin. After 30 minutes stay in a refrigerator the suspension was centrigufed for 5 minutes at 2500 rev/min. and inoculated intracerebrally (0.01 ml) and subcutaneously (0.03 ml) to one litter of 1 to 2-day-old suckling mice. To ascertain the mosquito virus level a suspension from 10 mosquitoes ground in 1 ml of diluent was titrated and each tenfold dilution was inoculated intracerebrally to suckling mice. The mosquito suspension was considered as a 10^{0} dilution. The virus level was expressed as the total average amount LD_{50} of virus in the body of one mosquito as was described earlier (DANIELOVÁ, MINÁŘ, ROSICKÝ 1968).

Observations on chimpanzees exposed to the mosquito feeding

Viremia: The chimpanzees were examined for viremia during 15 days after the exposure to mosquitoes at 24 hours' intervals. Samples of blood were obtained from the antebrachial vein or by cardiac puncture. For virus recovery the blood was taken into syringes rinsed with heparin solution, containing 100 I.U. of heparin in 1 ml of buffered saline, pH 7.2. The virus contents in the blood of chimpanzees was determined by the intracerebral inoculation of tenfold dilutions of blood into groups of 4—6 weanling mice weighing 7—6 g. Undiluted blood of the chimpanzees was also routinely inoculated intracerebrally to two litters of 1 to 2-day-old suckling mice in order to detect low levels of circulating virus. Virus concentrations were expressed as the number of LD_{50} per 0.03 ml of inoculum as calculated by the formula of REED and MUENCH (1938). The specificity of death of the mice was checked by subpassages or also by intracerebral virus neutralization tests in mice with immune mouse serum against Ťahyňa virus.

Physical examinations: The rectal temperature of each chimpanzee was taken during 14 days before and during 21 days after the exposure to mosquito bite twice daily. 36.9 °C were considered the upper limit of normal body temperature. Further daily examinations included auscultation

of the heart and respiratory system, palpation of the lymph nodes and observation of the animal's behavior. During the whole period of experiment the erythrocyte sedimentation rate was ascertained in 3 to 7 days' intervals. Fecal specimens or rectal swabs were collected at 7 days' intervals for bacteriological and parasitological examinations.

Antibodies: Sera from the chimpanzees collected before and at intervals for as long as 3 months after the exposure to the mosquito bite, were tested for the presence of neutralizing, hemagglutinin inhibiting and complement fixing antibodies to Ťahyňa virus. The sera were kept at —20 °C and all samples taken from one chimpanzee were examined simultaneously.

The virus neutralization test was carried out in the GMK AH-1 green monkey kidney stable cell line (GÜNALP 1965). The tube culture of GMK cells were grown in a synthetic medium (SLONIM at al. 1960) containing 10 % heated calf serum and antibiotics, its pH was adjusted by adding 0.5 ml of 7.5 % $NaCHO_3$ solution per 100 ml medium. In the maintenance medium the amount of serum was decreased to 2 % and 1.4 ml of 7.5 % $NaHCO_3$ solution per 100 ml was used. Undiluted sera and serial fourfold dilutions of the sera (inactivated at 56 °C for 30 minutes) were mixed in equal volume with 30—100 CPD_{50} of Ťahyňa virus. After incubation at room temperature for one hour the virus-serum mixtures were inoculated into two tube cultures for each serum dilution. The antibody titer was recorded as the highest serum dilution producing virus neutralization.

The hemagglutination inhibition test was carried out by the method of CLARCK and CASALS (1958). HA antigen was prepared from infected newborn mouse brains by the sucrose-aceton extraction, and the test sera were diluted in 2-fold steps starting from sera diluted 1 : 20. Four to eight hemagglutinating units were used.

In the complement fixation test carried out by the conventional method eight to sixteen units of antigen and two units of complement were used with an overnight incubation at 4 °C. The sera were diluted in two-fold steps starting with undiluted sera.

RESULTS

The transmission of the Ťahyňa virus to chimpanzees was performed 17 or 18 days after the infection of *C. annulata* mosquitoes. The virus titer of the mosquito infection source had been of $10^{4.4}$ LD_{50}.

The average virus level in one mosquito checked before and after feeding on chimpanzees reached the values of $10^{5.75}LD_{50}$ and $10^{5.29}LD_{50}$ respectively. At this time the infection rate in mosquitoes showed fifty per cent.

Chimpanzee No. 31 (a male weighing 4.5 kilograms) was exposed to 39 mosquitoes *C. annulata* out of which five specimens were found engorged after the exposure. The Ťahyňa virus was detected in three of them. Viremia was recovered in chimpanzee No. 31 on suckling mice inoculated with undiluted blood obtained on the first and second day but the amount of virus in the blood of chimpanzee on these days was too low to be titrated in weanling mice. The quantity of virus circulating during the third to sixth day after infection was higher and reached the maximum titer of $10^{2.0}LD_{50}$/0.03 ml of blood on the fourth postinfection day. The inoculation of undiluted blood into suckling mice failed to reveal the presence of viremia after the eighth day (Table 1).

On the second and fourth days of infection the chimpanzee No. 31 developed a rise of temperature. In the morning and in the afternoon of the second day 37.3 °C

Table 1. Viremia in chimpanzees following exposure to bite of *Culiseta annulata* infected with Ťahyňa virus

Chimpanzee No.	Virus titre in the blood (log. LD_{50}/0.03 ml)* on days after exposure to mosquito bite									
	1	2	3	4	5	6	7	8	9	10—15
31	<0.5	<0.5	1.0	2.0	0.5	0.6	<0.5	<0.5	0	0
33	<0.5	<0.5	<0.5	1.0	2.0	0.7	<0.5	<0.5	0	0
35	0	<0.5	1.0	0.5	1.5	>2.4	1.0	<0.5	<0.5	0
36	0	0	0	0	0	0	0	0	0	0
39	<0.5	0.5	<0.5	0	0	0	0	<0.5	<0.5	0

* 0 = virus recovered
<0.5 = virus isolated from undiluted blood in occasional weanling mouse or in suckling mice

was observed, by the third day the temperature returned to normal and in the afternoon of the fourth day again a rise to 37.6 °C was registered. On the following days the chimpanzee developed normal temperature (Fig. 2).

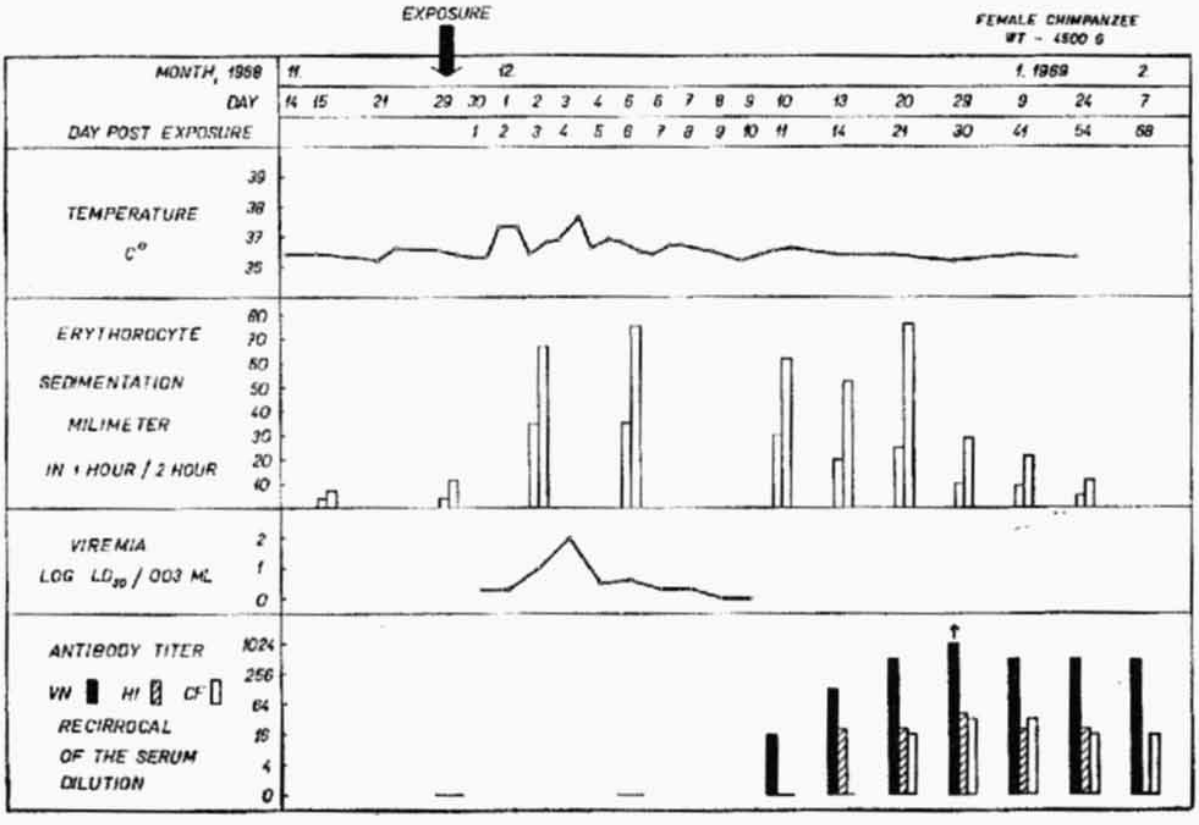

Fig. 2. Infection in chimpanzee No. 31 following exposure to bite of *Culiseta annulata* infected with Ťahyňa virus.

The erythrocyte sedimentation rate of this chimpanzee estimated prior to the exposure to the mosquito bite showed 4/7 and 4/12 millimetres respectively. On the third, sixth, eleventh, fourteenth and on the twenty-first postinfection days 35/67, 35/76, 30/62, 20/55 and 25/77 millimetres were observed. From the thirtieth

day a gradual return to the preinfection rate was established and on the 54th day after infection again 5/11 millimetres were found (Fig. 2).

The chimpanzee No. 31 possessed no demonstrable Ťahyňa virus neutralizing, complement fixing or hemagglutination inhibiting antibodies initially and also on the sixth postinfection day. The virus neutralizing antibodies appeared on the eleventh day, gradually reached high levels with a peak titer of >1024 on the thirtieth day and persisted for the whole period of investigation, i.e. for three months in a titer of 512. The hemagglutination inhibiting antibodies appeared on the fourteenth day after infection, a peak titer of 40 was observed on the thirtieth day but since two months after the virus inoculation by mosquitoes no hemagglutination inhibiting antibodies were found in the tested chimpanzee. The complement fixing antibodies were detected in this animal for the first time three weeks after exposure, they reached a titer of 32 at the fourth and sixth weeks and persisted in a titer of 16 even three months after infection (Table 2).

Table 2. Antibody response of chimpanzees following exposure to *Culiseta annulata* infected with Ťahyňa virus

Days post exposure	Chimpanzee														
	31			33			35			36			39		
	VN	HI	CF	VN	HI	CF	VN	HI	CF	VN	HI	CF	VN	HI	CF
0	0	<20	0	0	<20	−*	0	<20	0	0	<20	−*	0	<20	0
6–7	0	<20	0	0	<20	0	0	<20	−*	0	<20	−*	0	<20	−*
11–12	16	<20	0	8	<20	0	0	<20	0	0	<20	−*	4	20	−*
14–15	128	20	−*	1024	20	>2	1024	<20	8	0	<20	−*	512	20	−*
21–22	512	20	16	>1024	20	8	−**	−**	−**	0	<20	−*	256	20	−*
30–31	>1024	40	32	1024	20	−*	—	—	—	0	<20	−*	1024	40	32
41–42	512	20	32	1024	40	8	—	—	—	0	<20	−*	64	20	32
54–55	512	20	16	1024	40	8	—	—	—	0	<20	−*	64	20	32
68–69	512	<20	16	1024	20	4	—	—	—	0	<20	−*	64	20	16
92–93	512	<20	16	1024	<20	4	—	—	—	0	<20	−*	64	<20	16

VN, HI and CF titers represent the reciprocal endpoints of serum-dilution
0 no antibodies detected in undiluted sera
* Serum anticomplementary at dilution ≦1 : 16
** Animal died

In a second attempt at transmission of the Ťahyňa virus 41 mosquitoes *C. annulata* fed on the chimpanzee No. 33 (a female weighing 4.0 kilograms) exposed to 70 mosquitoes.

Viremia detected in this chimpanzee resembles the course of viremia in the chimpanzee No. 31 (Table 1).

On the second day of infection the chimpanzee No. 33 developed fever. That day in the morning a rise in temperature to 37.5 °C was observed, during the day the temperature continued to rise and in the afternoon reached 38.5 °C. On the following days the temperature dropped and on the fifth postinfection day only 35.5 °C

were registered. On the seventh day again a rise in temperature up to 37.2 °C appeared but since the eighth postinfection day a normal temperature was ascertained (Fig. 3).

The erythrocyte sedimentation rate showed an acceleration already on the third postinfection day when 40/80 millimetres were found in contrast with 5/15 and 5/17 millimetres observed prior to the exposure to infection. The sedimentation rate of erythrocytes in this chimpanzee showed a slow return to normal, on the 54th day 20/40 millimetres were yet ascertained (Fig. 3).

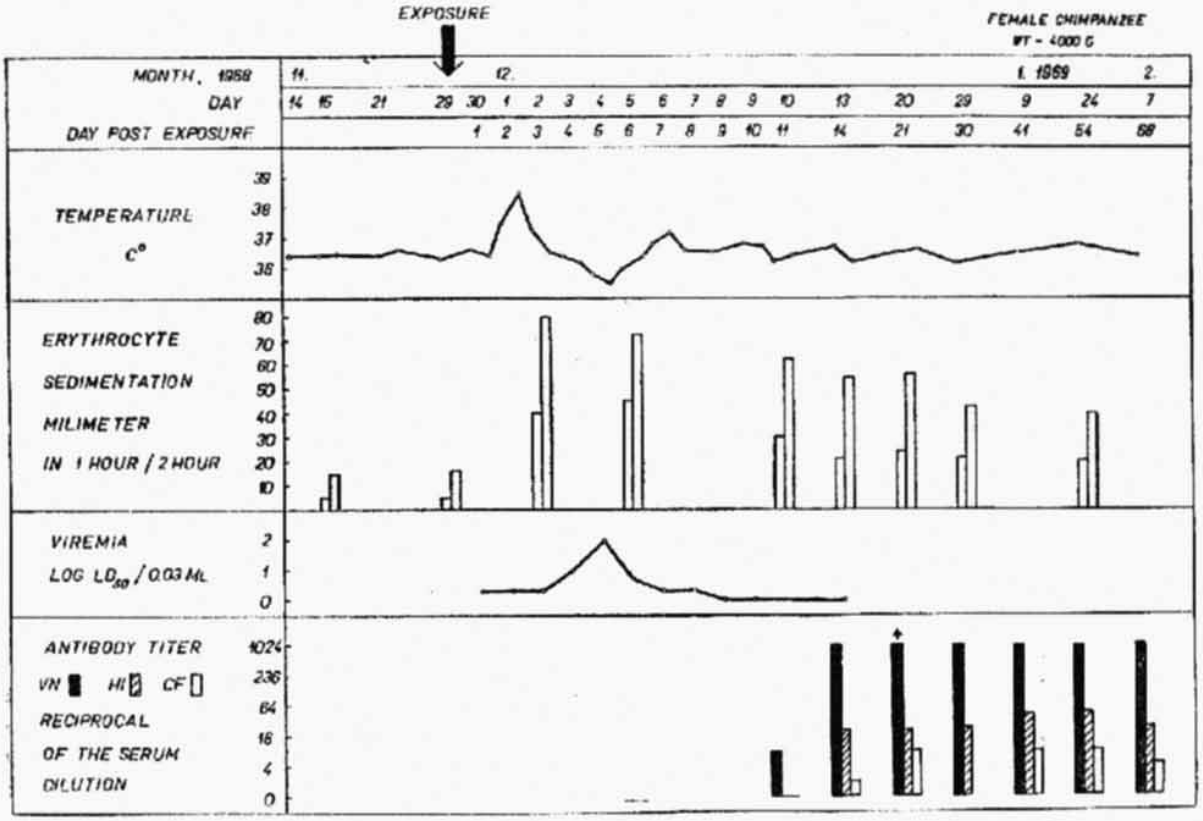

Fig. 3. Infection in chimpanzee No 33 following exposure to bite of *Culiseta annulata* infected with Tahyňa virus.

The antibody response of the chimpanzee No. 33 is given in Table 2. While on the eleventh postinfection day the virus neutralization antibodies showed a low level, on the fourteenth day already a titer of 1024 was found and this high level of antibodies persisted for the whole period of investigation without changes. On the fourteenth postinfection day also the hemagglutination inhibiting antibodies developed and reached a titer of 40 during the sixth and eighth weeks, then declined, two months after infection still a titer of 20 was found, but a week later the chimpanzee possessed no hemagglutination inhibiting antibodies. The complement fixing antibody formation showed a low level on the fourteenth day, and titers of 8 and of 4 persisted during the three months of investigation.

In three further experiments the chimpanzee No. 35 (a female weighing 4.1 kilograms) was exposed to 75 mosquitoes out of which 30 were found engorged, the chimpanzee No. 36 (a female weighing 4.7 kilograms) was exposed to 78 mosquitoes

out of which 37 fed on it and finally 8 mosquitoes were fed upon the chimpanzee No. 39 (a male weighing 4.1 kilograms) exposed to 53 mosquitoes.

While the chimpanzees Nos. 31 and 33 revealed no symptoms of disease during the last two-week period of quarantine, in the chimpanzees Nos. 35, 36 and 39 a raise in erythrocyte sedimentation rates was observed during the whole isolation period and in the chimpanzee No. 38 also temperature oscillations from normal to fever were found. For this reason in the chimpanzees Nos. 35, 36 and 39 no erythrocyte sedimentation rate and in the chimpanzee No. 36 neither the body temperature could be evaluated following the exposure to the Ťahyňa virus infection.

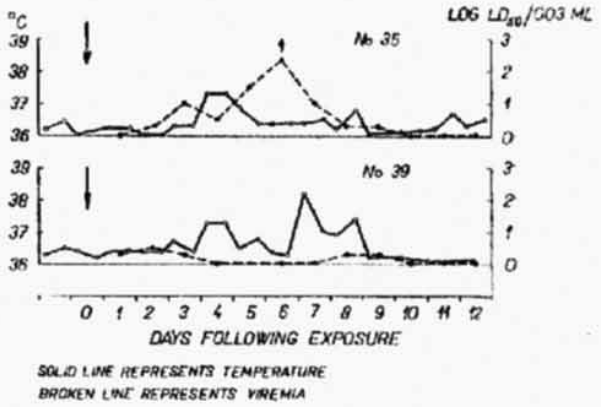

Fig. 4. Response of chimpanzees No. 35 and No. 39 to inoculation of Ťahyňa virus by infected mosquitoes.

Chimpanzees Nos. 35 and 39 developed viremia as well as antibodies following the infection but in the blood of the chimpanzee No. 36 no virus could be detected at any interval following the exposure to mosquito feeding and the results of serological examinations of this chimpanzee revealed no changes in comparison with the data obtained before the exposure to the mosquito feeding (Tables 1 and 2).

The course of temperature and viremia following the Ťahyňa virus infection of chimpanzees Nos. 35 and 39 is shown in Fig. 4. In the blood of the chimpanzee No. 35 no virus could be detected 24 hours after exposure. Traces of virus were found on the second day and the blood virus concentration reached the maximum on the sixth postinfection day, when a titer of $10^{>2.4}LD_{50}$/0.03 ml of blood was ascertained. Viremia lasted for eight days. A rise in temperature appeared on the fourth day following the exposure, when in the morning and in the afternoon 37.3 °C were registered. On the fifth postinfection day the temperature returned to normal. The chimpanzee No. 39 showed a low level of viremia on the first, second and third day. From the fourth to seventh postinfection day no virus could be detected in undiluted blood of this chimpanzee, but on the eighth and ninth postinfection days again traces of blood virus occurred. The first rise in temperature occurred on the fourth day following the exposure, reaching 37.3 °C in the morning and also in the afternoon. After a two days period of normal temperature on the seventh day fever of 38.3 °C appeared in the morning, but in the afternoon only 37 °C were ascertained. Next day in the afternoon the temperature reached once again 37.4 °C—and from the ninth postinfection day a normal temperature was observed.

None of the five chimpanzees, with exception of one animal, showed any marked changes in their behavior during the experiment. One chimpanzee, No. 35, was found to be noticeably weak when removed from the cage for examination on the 13th postinfection day. On this day still a normal temperature of 36.4 °C was ascertained in it. On the next three days this chimpanzee was sitting quietly in the cage, was

eating less and his body temperature dropped below 35 °C. On the 17th day following the infection the chimpanzee No. 35 died. The dissection examinations, including histological observations of organs revealed no pathological findings. No virus could be demonstrated in either part of the brain or striated muscles, lungs, spleen, liver, kidneys and inguinal lymph nodes.

DISCUSSION

The elucidation of clinical illness caused in humans by Ťahyňa virus is handicapped by the lack of virus isolation from acute human Ťahyňa virus infections. From non-human primates, examined following an experimental subcutaneous infection with Ťahyňa virus, chimpanzees have been found the most suitable for the observations of overt clinical signs of experimental Ťahyňa virus infection (Šimková, Bárdoš 1966).

The mosquito *Aëdes vexans* which is the main vector of Ťahyňa virus in Czechoslovakia (Šimková, Danielová, Bárdoš 1960; Danielová 1966), this could not be used because the experiments were carried out during the winter season. *Culiseta annulata* mosquito has been found a very suitable vector for multiplication and also for overwintering of Ťahyňa virus (Danielová, Minář 1969).

The transmission of Ťahyňa virus by the mosquito *C. annulata* obtained in four out of five exposed chimpanzees is the first proof of direct transmission of this infection to animals by *C. annulata* feeding on them.

The described experiments have demonstrated that chimpanzees may be infected by mosquitoes with Ťahyňa virus. The chimpanzees Nos. 31, 33, 35 and 39 each developed viremia and rise of temperature or fever, with following antibody formation. In two chimpanzees also the acceleration of erythrocyte sedimentation was observed. As for the relation between viremia and the febrile period, we found it interesting and important that the rise of temperature preceded the development of viremia in all four chimpanzees. Although caution must be exercised in drawing parallels between the responses in experimentally infected chimpanzees and naturally infected humans, the results of these studies might be useful in attempt to isolate virus from an acute human Ťahyňa virus infection. Further results of these studies suggest that the temporary appearance and persistence of Ťahyňa virus neutralizing, hemagglutinin inhibiting and complement fixing antibodies in the chimpanzees infected by mosquito feeding closely resemble those seen in human infections. Certain antibody responses seen in natural infections might well be explained by some of the antibody patterns observed in chimpanzees.

We did not succeed in proving by Ťahyňa virus isolation technique that the death of the chimpanzee No. 35 was specific, but this possibility was not excluded.

The failure in developing infection in the chimpanzee No. 36 is very interesting. However, the virus must have been inoculated to this animal during the mosquito feeding. 37 mosquitoes fed on this chimpanzee and even if the infection rate in *C.*

annulata was very low (only 50%) and the transmission rate which was unknown might be lower than the infection rate, we consider the number of feeding mosquitoes as sufficient. This conclusion is illustrated by the results obtained in the chimpanzees Nos. 31 and 39. In the chimpanzee No. 31 the illness occurred even after only three infected mosquitoes had fed on it.

The virological, immunological and clinical characteristics of Ťahyňa virus infections induced by mosquitoes in chimpanzees call attention to the potential usefulness of this model in studying human disease.

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