EXPERIMENTAL INFECTION OF SHEEP WITH BHANJA VIRUS

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Abstract. Seven juvenile sheep were infected subcutaneously (s.c.) or intracerebrally (i.c.) with Bhanja virus. Distinct symptoms of CNS affection (ataxia, paresis) were observed only in the case of i.c. application of a massive dose (10^6 to 10^7 SMID50) of the Bg 336/336 strain. A short-term, and low viremia (one to four days p.i.) was ascertained after i.e. infection with a dose of 10^2 to 10^3 SMID50 of the same strain. On the other hand, the high titres of neutralisation antibodies were detected even after inoculation of small doses of the virus (e.g. 6.0 SMID50). Unlike the symptomatic response, the immune response of sheep to Bhanja virus is high.

Pathogenicity of the recently discovered Bhanja virus (Shah and Work 1969) has been so far tested only in a few species of mammals (Balducci et al. 1970, Verani et al. 1970, 1971, Vesenjak-Hirjan et al. 1977, Hubálek and Rödl 1981) and birds (Semenov et al. 1973, Hubálek and Rödl 1980). For the time being there are no data available on the experimental or spontaneous pathogenicity of this virus to sheep, although antibodies are frequently found in these animals in natural foci (Verani et al. 1970, 1971, 1977, Albanese et al. 1971, Castro et al. 1976, Bárdoš et al. 1977, Vesenjak-Hirjan et al. 1977, Pavlov et al. 1978), and the virus was also isolated from the blood of sheep (Causey et al. 1969, Kemp et al. 1971). Camisas et al. (1981) described experimental infection of one sheep with Bhanja virus, but detected no viremia and neither did he observe any clinical symptoms.

MATERIAL AND METHODS

All merino sheep used in the experiments were two months old, weighing about 20 kg each. During each of the three experiments they were fed meadow hay and pollard and stalled in one pen with unregulated temperature ranging from about 18 to 25°C, and relative air humidity about 80%.

The animals were inoculated subcutaneously (above the right shoulder blade) or under thiopental anesthesia intracerebrally (in the left hemisphere) with 1 ml of inoculum in buffered phosphate solution (Dulbecco and Vogt 1954), pH 7.4 to which antibiotics and 0.75 % of bovine serum albumin (USOL, Praha) were added. Three strains of Bhanja virus were used for infection; they were isolated from the ticks of the genus Haemaphysalis (H. punctata, H. sulcata) and all prepared as centrifuged (15 min/1500 G) and adequately diluted suspensions of infectious brains of suckling mice (SMB); Bg 336 Ms or MsB, Bg 355/356 MsB (Pavlov et al. 1978) and ISS.BR 200 Ms (Verani et al. 1970), where x in MsB indicates the total number of passages in SMB. One ml of centrifuged (15 min/1500 G) 10 % suspension of normal non-infections SMB in PBS with 0.76 % of bovalbumin was applied to the control sheep. The dose of inoculated virus was ascertained by i.e. titration on sucking white mice before and after inoculation of sheep and expressed in SMID50, after Reed and Muench (1938). All inocula were bacterially sterile.

The examination of the presence of neutralizing (Nt) antibodies in sera before and after infection was carried out on test-tube cultures of Vero cells, with a constant dose of Bhanja virus Bg 336 and sera diluted fourfold (for details see Hubálek and Rödl 1981); before use all sera were inactivated for 50 min/56°C.

Viremia was determined by inoculating i.e. 1:3-day-old ICR mice with undiluted or slightly
RESULTS

Experiment No. 1. On 4 March 1980 the lamb A (a ram) was inoculated s.c. with a dose of $10^6.5$ SMLD$_{50}$ of Bhanja virus, strain Bg 326 (M4), the lamb B being a control. Viremia was not ascertained but seroconversion was found in the infected animal (Table 1). Its body temperature remained normal and no clinical symptoms were observed.

| Table 1. Experiment No. 1: s.c. infection with the strain Bg 326 (M4) |
|---|---|---|---|---|---|---|---|
| **Sheep** | **Sex** | **Dose of virus SMLD$_{50}$** | **Viremia (days p.i.)** | **Reciprocal NT titre (days p.i.)** |
| | | 2 | 4 | 5 | 7 | 9 | —1 | 24 | 70 | 96 |
| A | ♀ | $10^5.4$ | 0/11 | 0/10 | 0/10 | 0/10 | 0/11 | < 4 | 256 | 512 | 8192 |
| B | ♀ | 0 | 0/9 | 0/8 | 0/10 | 0/10 | 0/8 | < 4 | < 4 | < 4 | 64 |

1. The numerator indicates the number of specifically dead mice, the denominator — total number of suckling mice inoculated i.e.
2. On day 70 p.i., the sheep A and B were challenged i.e. with the dose of $10^6.5$ SMLD$_{50}$ of the Bg 326 (M4) virus.

On day 70 p.i. the two lambs were inoculated s.c. with the same strain of the virus in identical SMB passage, the dose being $10^6.5$ SMLD$_{50}$. In lamb A a considerable increase of the antibody titre (booster effect) was observed, in lamb B a seroconversion took place after challenge. The body temperature rose slightly in the first two days p.i. afterwards it dropped again. No pronounced clinical symptoms were observed after challenge.

Experiment No. 2. On 3 December 1980 three young sheep were inoculated i.c. with three strains of Bhanja virus (all as 20 % SMB). In all infected animals seroconversion took place (Table 2).

| Table 2. Experiment No. 2: i.c. infection with three strains of the Bhanja virus |
|---|---|---|---|---|---|---|---|
| **Sheep** | **Sex** | **Virus and passage** | **Dose of virus SMLD$_{50}$** | **Viremia (days p.i.)** | **Reciprocal NT titre (days p.i.)** |
| | | 2 | 5 | 7 | 0 | 33 | 110 | 204 |
| C | ♀ | ISS.IR.205 (M56) | $10^6.4$ | 0/22 | 0/17 | 0/16 | < 4 | 256 | 512 | NT$^2$ |
| D | ♀ | Bg 326 (M4) | $10^6.4$ | 0/23 | 0/24 | 0/9 | < 4 | 1024 | 1024 | NT$^2$ |
| E | ♀ | Bg 335/336 (M4) | $10^6.2$ | 7/16 | 0/15 | 0/17 | 4 | 4096 | 8192 | 2048 |
| F | ♀ | 0 | NT | NT | NT | < 4 | 4 | 32 | NT |

1. see Table 1 2. not tested.

Viremia was demonstrated 48 hours p.i. in the sheep E infected with the virus Bg 335/336. Its body temperature rose above 40°C, reaching the maximum of 41.7°C 48 hours p.i.; on the third day p.i. it dropped below 40°C, but another increase above 41°C was recorded on day 6 p.i. (Fig. 1). From the third day p.i. pronounced clinical symptoms were observed in the animal, evidencing CNS disorder. At first, short cramped-like fits appeared at irregular intervals. About 40 hours later the fits were replaced by paroxysms of the posterior part of body. The animal was lying sideways and could not move. Three days later the paroxysms gradually receded, the lamb repeatedly stood up, but after a few steps collapsed again, grit its teeth and propped its head against the wall. Increased respiration frequency and shedding of wool was observed, but the animal fed normally. These symptoms disappeared within 12 days after their appearance. The blood count of the experimental animal was also considerably changed. On day 5 p.i. the number of segmented leukocytes was more than double (raising from 33% to 77.5%), while the percentage of lymphocytes dropped from 56.5% to 18%.

The highest titre of NT antibodies was ascertained in this animal, persisting until day 334 p.i. (the period of studies). In animals C and D no symptoms of CNS disorder were observed, there was only temporary weakness and a slightly risen temperature on days 2–3.

The control animal (F) showed a risen temperature on day 1–2 p.i. (unspecific pyretic reaction due to operation i.e. inoculation of normal 20 % SMB with 0.75 % bovaalbumin, i.e. antigenically active substances). Seroconversion appeared reaching a low titre (1:28) on days 33–110 p.i.; this animal had been apparently infected by contact during the long-term joint stalling with the infected sheep.

Experiment No. 3. On 14 May 1981 three sheep were inoculated i.c. with different doses of virus Bg 335/336 (M5). Seroconversion took place in all animals, including the sheep G, inoculated with 50 SMLD$_{50}$ (Table 3). A light viremia was detected only in animals H and I 24 hrs p.i., and moreover in the sheep H also on day 4 p.i.

| Table 3. Experiment No. 3: i.c. infection with different doses of Bg 335/336 (M5) virus |
|---|---|---|---|---|---|
| **Sheep** | **Sex** | **Dose of virus SMLD$_{50}$** | **Viremia (days p.i.)** | **Reciprocal NT titre (days p.i.)** |
| | | 1 | 4 | —1 | 42 |
| G | ♀ | $10^6.65$ | 0/17 | 0/17 | < 4 | 512 |
| H | ♀ | $10^6.65$ | 2/19 | 1/18 | < 4 | 512 |
| I | ♀ | $10^6.65$ | 1/18 | 0/19 | < 4 | 1024 |

1. see Table 1
Clinical symptoms of CNS origin were observed only in the lamb I, which had been infected with the highest dose of virus. The symptoms appeared on day 5 and were characterized by weakness of the posterior part of body inducing the animal to lie down for one day, by unphysiological posture with drooped head and by ataxia. Similarly as in the previous case, the lamb recovered fully after a few days. After inoculation the temperature of this animal rose to 41.5°C during viremia (on day 1 p.i.). A further rise of temperature above 40°C was observed on days 3 and 4 p.i.; the temperature dropped afterwards.

**DISCUSSION**

The clinical manifestation of the infection with Bhanja virus in the distinct form (also, a rare paresis) was observed in the sheep only if i.e. application of a massive dose (10⁵ SMicLldo) of the strain Bg 335/336. Despite this the animal (E) recovered spontaneously. Less pronounced symptoms of CNS affection were also observed in animal I, which had been infected with a lesser dose (10⁴.5 SMicLldo) of the same strain; when lower doses of the virus, or other strains, were used, no similar symptoms were recorded. The virus was present in the blood of sheep p.i. only when the strain Bg 335/36 was used, at low titres and for a short period. The highest level of viremia (ca. 40–50 SMicLldo/ml of blood) was detected in the sheep E 48 hrs p.i. It should be noted, however, that for technical reasons the blood samples could not be examined on viremia immediately, but only after 2–20 day storage at −60°C. This procedure, including freezing and thawing of blood, is not optimal for the detection of any trace of viremia amount, of course. Moreover, we did not test the blood on the virus presence every day p.i., but at 2–3 day intervals.

However, the sheep are immunologically unusually susceptible to Bhanja virus: even low infectious doses (e.g. 50 SMicLldo in the animal G) were enough to produce an immune response and formation of relatively high levels of Nt antibodies which persisted for a long time (at least several months; cf. the sheep E) without any pronounced drop of titre. We also observed antibody response in the sheep E, induced probably by a mere contact with infected animals, this phenomenon being unusual in arboviruses. The characteristic of the virus (antigen) excretion in sheep and the possible mode of transmission were not studied. The high antibody levels against Bhanja virus were revealed during serological screening of sheep and goats in the west from the village Verani and in the village Vezenjak-Hirjan. We also witnessed the experimental infection of goats with this virus (Verani et al. 1970, 1971). On the other hand, in our previous experiments we found a considerably lower production of NT antibodies against Bhanja virus in rabbits and birds (HUBÁLEK and RÖDL 1980, 1981) than in sheep.

Based on the fact that the hosts' susceptibility to most arboviruses depends on the host age, it may be supposed that the pathogenicity of Bhanja virus for young lambs is considerably higher than for juvenile or adult sheep. It may also be surmised that under natural conditions the infection might be potentialized in juvenile and adult sheep under following circumstances:

1. massive infestation and intoxication of sheep by ticks;
2. immunosuppression due to different factors;
3. co-infection with another potentially pathogenic agent.

In addition, the genetic resistance of macroorganism (i.e. strain), sex, method of virus passage as well as individual virulence of the strain, may very much affect the course of infection with many arboviruses in sheep (GHALASAI et al. 1978, TOMORI 1979). Obviously, it will be necessary to study a number of variables before

Bhanja virus could be designated as low-pathogenic or nonpathogenic for juvenile or adult sheep. Our results rather indicate that the agent under certain circumstances may cause traumatic infections in sheep or even epizootics in natural foci.

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**ЭКСПЕРИМЕНТАЛЬНОЕ ЗАРАЖЕНИЕ ОВЕЦ ВИРУСОМ БАНДИКА (БХАНЖА)**

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Резюме. Семь молодых овец заражали подкожно в вену в мох вирусом Банжика. Явные симптомы поражения ЦНС (атаксия, парезы) наблюдали только в случае введения в мох массовой дозы (10⁵–10⁶ SMicLldo) штамма Bg 335/36. Но некоторым животным после введения малой дозы (10⁴–10⁵ SMicLldo) того же штамма. С другой стороны, высокие титры нейтрализующих антител обнаружены даже после введения небольших доз вируса (например, 50 SMicLldo). Иммунная реакция овец на вирус Банжика, в отличие от симптоматической, высока.

**REFERENCES**


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