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The serological response and long-lasting resistance against infection with louping-ill virus in sheep immunized with a highly attenuated tick-borne encephalitis virus

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Infections with louping-ill (Li) virus, belonging to the tick-borne encephalitis (TE) complex of the B group of arboviruses, represent an important veterinary and human public health problem, mainly in Scottish natural foci of Li, in northern England and in Ireland. The Li virus, transmitted by the tick *Ixodes ricinus*, causes not only economic losses in flocks of sheep, but is known also as an aetiological agent in human infections.

To protect sheep against Li, intensive studies of the immunizing effect of a formolized virus vaccine were undertaken (Edward, 1947; Williams & Thorburn, 1961). The use of the live TP-21 strain (Smith, 1956) of the Malayan Langat virus, showing greatly lowered virulence for sheep, is not recommended by O'Reilly *et al.* (1965) because of a weak antibody response.

In the course of the investigation of immunogenic and antigenic properties of the monkey- and mouse-attenuated clone, designated Hy-HK 28 '2', of the TE (Western subtype) virus, a distinct protective effect in immunized animals against challenge with various virulent strains of different members of the TE complex was observed. Even a single dose of the attenuated TE virus, administered subcutaneously, elicited in large domestic milk-giving animals seroconversions into positivity accompanied by a high and long-lasting resistance (Mayer *et al.* 1967; Blaškovič *et al.* 1969). This marked immune response was observed mainly in goats, animals important in the epidemiology of milk-transmitted, human alimentary infections with the TE virus.

The aim of the present study was to investigate the degree of the resistance which develops in sheep, immunized with a single dose of the attenuated TE virus. These experiments were also intended to study the specific immune response, including the persistence of resistance to challenge with virulent Li virus in a long-term study. The investigations of the influence of the time factor on the degree of the resistance developed were expected to show to what extent the Hy-HK 28 '2' virus, differing in the character of at least 8 genetic markers (Mayer, 1966; Mayer & Rajčáni, 1968) from the usually encountered highly virulent TE virus strains, is capable of eliciting a long-persisting immune state in domestic animals. In this study it concerns sheep, which under natural conditions participate in the ecological cycle of TE and similarly also of Li virus.

The work presented was performed as a part of an extensive field trial of experi-

mental immunization of domestic milk-giving animals (goats, cattle and sheep) using the attenuated TE virus, with the final aim to collect information regarding the possibility of preventing alimentary human TE infections. Results of this field trial are described in detail elsewhere (Blaškovič *et al.* 1969).

MATERIALS AND METHODS

Viruses

For immunization of sheep, the virus clone, designated Hy-KH 28 '2', derived from the prototype Czechoslovak strain 'Hypr' of the TE virus (Western subtype) (Pospíšil, Jandásek & Pešek, 1954) was used. This particular clone, described in detail elsewhere (Mayer, Slavik & Libíková, 1967*a*) is avirulent for 6–8 g. white mice (Děčín breed) after subcutaneous inoculation and for young *M. mulatta* and *M. radiata* monkeys after intrathalamic or intranasal administration (Mayer & Rajčáni, 1967; 1968). The virus was used in the form of 10 % mouse brain suspension with the titre of $10^{8\cdot0}$LD 50/ml. after intracerebral and $10^{1\cdot0}$LD 50/ml. after subcutaneous inoculation of young mice.

For challenge of immunized sheep and infection of control, non-immune animals the 'Li 1959' strain of the Li virus (from the collection of viruses, Institute of Virology, Bratislava, strain originally supplied by Dr McCallum, London) was used. This virus strain was originally isolated from the brain of a sick lamb and underwent 7 intracerebral passages. The virus titre in 10 % mouse brain suspension was $10^{8\cdot2}$/ml.

Animals

Ninety-seven sheep, 1–2 years old, were used in this study. These animals, from which 74 were used for the immunization experiment and 23 served as controls (placebo), belonged to one herd, grazing on the southern slopes of Tribeč Mountains (south-west Slovakia), a locality known as a natural focus of TE.

Before the experiment 11 sheep from the larger group of animals (14·8 %) and 6 from the 23 control animals (26 %) showed the presence of virus-neutralizing antibodies (VNA) against TE virus in their serum (Tables 1, 2). As control for the challenge experiments, 4 animals of the same age as the immunized sheep, but without detectable VNA, were chosen.

Viraemia

During the first days after the challenge, the blood from all animals was assayed for the presence of the virus. The isolation experiments were performed by intracerebral inoculation of mice. Each blood sample was administered immediately after collection into one litter of 8–10 newborn mice. Virus in ill mice of the primary passage was demonstrated definitely by means of a second passage. The isolated virus was identified by the virus-neutralization test using specific immune serum. Blind passages were performed on the 10th day after infection by inoculating a 10 % mouse brain suspension from primary passage into further litters of suckling mice. The intracerebral route was invariably used.

Virus-neutralization tests

Titres of specific VNA in sheep sera were determined in cell cultures of Detroit-6 cells and Salk cynomolgus heart cells using a cytopathic variant of TE virus (Libíková, 1963). The initial dilution of the inactivated serum was 1/4. Blood samples were collected at intervals from the jugular vein. Separated sera were stored frozen until they were examined.

Table 1. *Virus-neutralizing antibody in sheep before immunization, and 6 weeks, 9 months and 12 months after immunization with one dose, containing 10^7 mouse ICLD 50, of attenuated tick-borne encephalitis virus*

	Before immunization		After immunization					
			6 weeks		9 months		12 months	
Antibody titre	No. of sheep	(%)	No. of sheep	(%)	No. of sheep	(%)	No. of sheep	(%)
< 4	63	85	9	13	3	5	3	6
4	10	14	21	30	15	24	14	27
8	0	—	22	31	21	34	16	31
16	1	1	16	23	12	20	10	19
32	0	—	1	1	7	11	7	13
64	0	—	1	1	1	2	0	—
⩾ 128	0	—	0	—	2	3	2	4
Total no. of animals	74	—	70	—	61	—	52	—
Antibody present in	11	15 %	61	87 %	58	95 %	49	94 %

Table 2. *Virus-neutralizing antibody titres in the control group of sheep before and 6 weeks and twelve months after administration of placebo*

	Before placebo		6 weeks after placebo		12 months after placebo	
Titre of virus-neutralizing antibodies	No. of sheep	(%)	No. of sheep	(%)	No. of sheep	(%)
< 4	17	74	13	76	11	78
4	3	13	3	18	2	14
8	2	9	1	6	1	7
16	1	4	0	—	0	—
32	0	—	0	—	0	—
Total number of animals	23	—	17	—	14	—

RESULTS

Experimental immunization of sheep

Seventy-four 1- to 2-year-old sheep were subcutaneously injected with one 1 ml. dose of the attenuated Hy-HK 28 '2' virus, in the form of 1 % infected mouse brain suspension, containing 10^7 mouse ICLD 50. The remaining 23 sheep were

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given, also subcutaneously, one dose of 1 % normal mouse brain suspension, which served as placebo.

The evaluation of the whole experiment showed a striking immune response to TE virus in animals given the Hy-HK 28 '2' clone. The application of this clone stimulated the production of specific VNA to virulent TE virus to such an extent that specific VNA were observed 6 weeks after the injection in 87 % of sheep, including those where VNA were detected before the immunization (15 %). Nine or 12 months after the administration of the virus, the incidence of VNA reached 94 %.

VNA at titres of 1/4–1/16 were present 6 weeks after immunization in 84 % of sheep, but they were not detected in 13 % of animals. Nine months after immunization, VNA were absent in only 5 % of 61 sheep examined, and in 90 % of the animals they reached titres of 1/4–1/32. In two sheep VNA were found at a titre as high as or higher than 1/128. Similar serological results were also found with sera collected during the 12th month after immunization, when VNA titres of 1/4–1/32 were found in 90 % of the animals examined and the same two sheep as before showed the high titres ($\geqslant$ 1/128) (Table 1).

Table 3. *Immune response in sheep on the 6th week after administration of attenuated tick-borne encephalitis virus*

Effect of immunization on virus-neutralizing antibody	Number of sheep*	(%)
Seroconversion from negative to positive	50/59	85
Seronegative after immunization	9/59	15
Pre-existing antibody titres increased	9/11	82
Pre-existing antibody titres not increased	2/11	18

* Numerator = no. of animals showing effect; denominator = no. of animals examined.

When considering the type of immune response (Table 3), it is interesting to note that the serological conversion from negative to positive and the increase of the pre-existing VNA titres of the VNA was observed in approximately the same percentage, i.e. in 85 and 82 %.

From the 23 placebo-injected sheep only 14 remained to the end of the 12th month. From them only 3 animals showed the presence of VNA (Table 2). In this number is included also 1 sheep, in which a clear seroconversion to positive was observed.

Challenge of the one-shot immunized sheep

Four immunized sheep were selected on the basis of their VNA titres. Care was taken to choose animals which had rather low VNA titres, i.e. titres most frequently observed after immunization with the live virus. The selected sheep and three further control animals (without specific serum antibodies) were challenged with 10^5 ICLD 50 of virulent Li virus administered subcutaneously in the form of diluted 10 % mouse brain suspension.

Table 4. *Viraemia and virus-neutralizing antibody in sheep immunized with one dose of* 10^7 *ICLD*50 *of attenuated tick-borne encephalitis virus clone HY-HK* 28 '2', 324 *days before challenge with* 10^5 *ICLD*50 *of virulent louping-ill virus, and in control unimmunized sheep similarly challenged*

Sheep no.		Before challenge	Days after challenge: 2	3	4	5	6	7	8	9	10	11	12	14	16	21
657	Imm.*	4	–	–	–	–	–	–	–	–	–	–	–	.	.	.
			8	–	128	.	128	.	.	256	.	.	256	256	256	256
604	Imm.	4–8	–	–	–	–	–	–	–	–	–	–	–	.	.	.
			4–8	.	4–8	.	32	.	.	64	.	.	128	128	256	256
607	Imm.	4–8	–	–	–	–	–	–	–	–	–	–	–	.	.	.
			4–8	.	4–8	.	32	.	.	64	.	.	128	128	128	128
623	Imm.	8	–	–	–	–	–	–	–	–	–	–	–	.	.	.
			16	.	32	.	128	.	.	128	.	.	128	256	256	256
701	Cont.*	< 4	–	–	+	+	+	–	–	–	–	–	–	.	.	.
			< 4	.	< 4	.	4	.	.	16	.	.	16	16	16	16
711	Cont.	< 4	–	+	+	+	+	–	–	–	–	–	–	.	.	.
			< 4	.	< 4	.	< 4	.	.	8	.	.	8	8	4	4
732	Cont.	< 4	< 4	.	< 4	.	8	.	.	64	.	.	64	64	64	64

* Imm. = immunized. Cont. = control unimmunized animal.
The upper line for each animal shows viraemia. – = virus not detected in blind passage on unweaned mice. + = virus detected.
The lower line shows reciprocals of the neutralizing antibody titres.

Edward (1947) reported that the administration of 10^4–10^6 mouse LD 50 of the Li virus caused death in 66 % of sheep inoculated. The challenge was performed in January, as advised by the same author since the winter is claimed to be the most favourable season for the establishment of experimental infection.

During the first days after the challenge, the blood from all animals was assayed for the presence of virus. In the sheep immunized with the Hy-HK 28 '2' virus, no viraemia was observed between the 2nd and 12th day after challenge with louping-ill virus, irrespective of the actual level of VNA. In control previously non-immunized sheep, viraemia started on the 3rd–4th day after challenge and lasted for as long as 4 days. These results indicate the multiplication of the virus in the organism of non-immune animals (Table 4).

Furthermore, a very marked difference between the challenged immunized and control sheep was observed in their VNA titres. In immunized animals the titres of VNA increased considerably during 2–3 days after the injection of the challenging virus and, starting from the 6th day, they reached relatively high titres which were further maintained. High titres of specific VNA were observed in all immunized animals (Table 4). The immune response in control animals was considerably less pronounced. They developed VNA only later and in lower titres.

The temperature response and clinical observation revealed no significant differences between the immunized and control animals.

DISCUSSION

The results of experiments described above indicate that even a single dose of the monkey-attenuated virus was able to elicit in sheep an immune response lasting for at least 11 months. This immune response, although caused by the TE virus (Western subtype), was specific also against the serologically related louping-ill virus. The investigations of VNA showed that animals immunized with the attenuated virus acquired a considerable resistance against infection with a virulent virus, even when humoral antibodies were present at rather low titres. The relatively low VNA titres were observed also in goats, which similarly exhibited a high resistance against the challenging virus (Mayer *et al.* 1967*b*).

The steep increase of VNA titres observed during the first days after challenge of immunized animals, and the fact that this good anamnestic response (together with a satisfactory degree of resistance) was observed even when the challenging virus was administered on the 324th day after the one-shot immunization, seems to indicate that a highly monkey- and mouse-attenuated virus, as exemplified by the Hy-HK 28 '2' clone, could also serve as a good antigenic stimulus for sheep.

These experiments, which have rather an informative character, will need to be completed on a larger number of challenged animals.

The increase of the percentage of serologically positive sheep (from 87 to 94 %) and of the animals with the higher VNA titres, in the period between 6 weeks and 9 months after immunization, may be due either to contact of the sheep with virus circulating in nature, or to a slower immune response of some sheep to the administration of the attenuated virus. The hypothesis concerning the supposed contact

of animals during the spring and summer period with the virus circulating under natural conditions seems to be, in the majority of cases, the more probable, because the grazing locality is known as a natural focus of TE. This assumption is supported also by the relatively high number of sheep showing the presence of specific VNA before immunization. Thus it seems that the immunity conferred by immunization could be maintained or even increased by small booster doses of virus transmitted by ticks, parazitic on sheep.

The results obtained indicate that the immunization of sheep, raised and moving freely in geographic areas known as natural foci of TE or Li, could exert its effect at various levels. By preventing the detectable multiplication of the tick-transmitted virus in the organism of vaccinated animals (as shown by the absence of viraemia) the immunization (*a*) protects the sheep against clinically apparent forms of Li and (*b*) hinders the infection of other, still non-virophoric ticks, simultaneously infesting the same host animal, on which the infectious, virus-transmitting ticks are parasitic.

Thus the vaccination of sheep could be considered also as a limiting factor in the spread of the virus in nature, which has epizootiological and epidemiological implications.

SUMMARY

The vaccination of sheep with one dose of the monkey- and mouse-attenuated tick-borne encephalitis virus (the Hy-HK 28 '2' clone) causes seroconversion from negative into positive in 85% of animals. In sheep with pre-existing virus-neutralizing antibodies an increase of their titres was observed in 81%. The antibodies persisted for at least 12 months after the vaccination and during the summer period of grazing the number of serologically positive animals even increased.

The vaccinated animals, in contrast to the non-immune control sheep, developed no viraemia after challenge with the virulent louping-ill virus, performed 11 months after immunization.

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