Original Article

Preclinical tests of Vaccine against Rhinopneumonia and Strangles of young horses

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Abstract

Efficacy and safety of the combined vaccine against rhinopneumonia and strangles of horses were evaluated on outbred mice and rabbits. Acute toxicity, pyrogenicity and immunogenicity were carried out in accordance with the methods regulated by the Rosselkhoznadzor of the Russian Federation

The absence of toxicity was established with a single intragastric administration of the vaccine to white mice. An analysis of the dynamics of the body weight of white mice revealed an increase in both the control and experimental groups. Thus, the increase was 9.2% in the experimental group that received the vaccine preparation, and in the control group - 5.05%. Therefore, in mice treated intragastrically with the vaccine preparation, active growth is observed in comparison with the control. The difference was 4.15%. The disease and mortality among vaccinated mice were not noted, which indicates its harmlessness and safety.

The test of the vaccine preparation on rabbits showed its non-pyrogenicity. In the third hour after the administration of the drug, the temperature of rabbits in the control group rose by 0.2ºC, and in the experimental group by 0.4ºC. However, during a clinical examination of the experimental animals of both groups, signs of illness and changes in the general condition were not recorded. Thus, the studied vaccine preparation was considered non-pyrogenic, since after its administration to rabbits, a slight short-term increase in body temperature is observed in animals of both groups, in which the general condition of the animals remains favorable.

The immunogenicity of the viral component of the associated vaccine against rhinopneumonia and strangles of horses was studied on outbred mice. Studies have shown sufficient immunogenicity (75%) when the vaccine is administered subcutaneously at a dose of 0.3 cm3 twice with an interval of 14 days, followed by control infection with an adapted strain of equine rhinopneumonia virus at a dose of 0.02x6.0 lgTCD50/ml intracerebral.

Evaluation of the immunogenic activity of the strangles composition of the vaccine was carried out in experiments with a single immunization of white outbred mice, followed by infection with a virulent culture of the strangles streptococcus Streptococcus equi "H-5/1" at a dose of LD50 (200 thousand microbial bodies per 1 cm3). The bacterial component protects against the causative agent of strangles by 80% of the number of infected animals.

The aim of our research was to study the efficacy and safety of the combined vaccine against rhinopneumonia and strangles of horses in laboratory conditions.

This paper presents the results of preclinical studies on laboratory animals. The absence of acute toxicity, allergenic, pyrogenic properties and the safety of using the associated vaccine against rhinopneumonia and strangles of horses on laboratory animals have been proven.

Keywords: combination vaccine, acute toxicity, pyrogenicity, immunogenicity, horse strangles, rhinopneumonia, immunomodulator.

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1. INTRODUCTION

Viral and bacterial diseases of young horses represent a complex problem for the world horse breeding, which is still far from being solved. In the Russian Federation, in the Republic of Sakha (Yakutia), special attention should be paid to diseases of young horses, since these diseases cause enormous economic damage to herd horse breeding, which consists of a lack of offspring, culling of horses, loss of reproductive ability of mares, death of animals, as well as costs of medical and preventive measures. The studies of foreign authors and our previous studies have established that young animals up to one-year-old can get sick with several infectious diseases at the same time [1,2]. The most common diseases among young horses are rhinopneumonia and strangles of horses.

Rhinopneumonia is caused by the equine herpesvirus of the family Herpetoviridae, subfamily Alphaherpesvirinae. The disease can occur in several forms, ranging from a mild respiratory form to a more severe abortive and neurological form [3,4]. In animals infected with strangles, several forms of the disease can also be observed, of which the metastatic form is considered the most dangerous [5,6].

As mentioned above, rhinopneumonia and horse strangles are widespread throughout the world. Thus, in recent years, the rhinopneumonia virus has been detected in Israel [7], Australia, China and Ireland by a number of authors from different continents [8,9,10]. Strangles of horses is most common in Novosibirsk, Altai, Irkutsk regions, Krasnoyarsk and Altai territories [11], the Republics of Tyva [12], Khakassia, Sakha (Yakutia) [1] of the Russian Federation, as well as in Kazakhstan [13], Mongolia [14], Kyrgyzstan [15], the Netherlands [16], the Arab Republic of Egypt [17], Korea [18], Brazil [19].

According to the veterinary service of the Republic of Sakha (Yakutia) and our own research in 2002-2020 circulation of the rhinopneumonia virus was noted in 99 points of 16 districts of the republic, and the incidence of strangles of young horses is recorded annually and reaches 62.7% of the total number of young horses [1].

Currently, one of the effective, low-cost and safe measures to combat rhinopneumonia and strangles of horses is timely vaccination [13,20,21]. In the world there are different types of vaccines against rhinopneumonia: inactivated, attenuated, live, as well as different methods of therapy [10,22]. In Kyrgyzstan, an inactivated vaccine against rhinopneumonia is being developed from the isolate of “Kordai” virus serotype 4 [23]. In the Republic of Kazakhstan, vaccines against strangles, rhinopneumonia, and salmonella abortion have been developed and are being used [13]. Also, research is underway to develop a technology for manufacturing a vaccine preparation from the laboratory strain "AK-2011" of the equine rhinopneumonia virus [24]. In foreign countries, including Poland, France, and Egypt, various inactivated, live, mono- and polyvalent vaccines against rhinopneumonia are used [25]. These vaccines are inactivated and contain an adjuvant. The quality control of the vaccines showed that they do not contain foreign impurities and are safe for laboratory animals. Clinical trials have shown sufficiently high efficacy and safety. However, the technology for the use of these vaccines provides for two and three times immunization. [26,27,28]. Vaccines are not registered in the Russian Federation, except for the “Pneumequin” vaccine [21]. In Russia, a dry live virus vaccine from the CB/69 strain is used to prevent rhinopneumonia [29].

Some developers suggest using a combined vaccine against rhinopneumonia and equine influenza [30]. France allows the use of various mono- and polyvalent inactivated vaccines against rhinopneumonia and influenza [21]. However, these drugs are not registered in Russia. The technology of their application also provides for 2-3 times the introduction of the drug. It should be noted that equine influenza is not common in all regions of the Russian Federation, including Yakutia. In influenza-free areas, the use of this vaccine is not advisable.

In herd horse breeding, inactivated single-use vaccines have found wider use. Vaccines with double and triple use are not technologically suitable for prophylactic immunization of horses in the Far North [1,20]. In horse breeding farms of the Republic of Sakha (Yakutia), the use of vaccines with two and three injections is difficult due to natural and climatic conditions, since the second and third immunization of horses occurs in January-February. During this period, the ambient temperature reaches up to –55°C [1,29].

In 2000, employees of the Yakut Scientific Research Institute of Agriculture developed and introduced into production an inactivated vaccine against strangles of horses. However, this vaccine was discontinued due to the expiration of the registration period, and the strain from which the vaccine was made has lost its specific properties [1]. In 2018, a new strain of strangles streptococcus, Streptococcus equi H-5/1, was deposited at the FGBU VGNKI (registration number VKShM -B-141P). This strain was isolated from the washout of the opened submandibular lymph node of a sick foal from the village Nemyugyuntsy of the Khangalassky district of the Republic of Sakha (Yakutia) in 2016. At present, the Streptococcus equi H-5/1 strain is used to develop a vaccine against horse strangles (patent for invention №2703485 from 15.08.2018).

In the Republic of Sakha (Yakutia), employees of the Yakut Scientific Research Institute of Agriculture and VIEV (Moscow) jointly developed an inactivated vaccine from the CB/69 strain of equine rhinopneumonia with an immunomodulator from the fugat of the Bacillus subtilus strain TNP-3. The inactivated vaccine with a single vaccination of pregnant mares increased the business yield of foals by 10.9-33.3%. Also, immunobiological studies have confirmed the possibility of transmission of antibodies to the rhinopneumonia virus through colostrum from mother to foal from vaccinated mares [1].

We have established that rhinopneumonia has immunosuppressive abilities, which complicates the course of strangles. The simultaneous course of rhinopneumonia, strangles and salmonellosis in young horses has been proven [1]. In view of the fact that rhinopneumonia can occur simultaneously with strangles, the development of a bivalent combined vaccine against rhinopneumonia and strangles of horses, which provides for a single immunization against two diseases, remains relevant.

In the literature available to us, there are no data on analogues of a bivalent vaccine for the prevention of rhinopneumonia and

strangles of horses. The introduction of different vaccines in a limited period of mass treatments in herd horse breeding is a laborious undertaking. The simultaneous administration of different immunobiological preparations is prohibited by veterinary legislation.

Thus, it is of interest to conduct preclinical trials of an inactivated associated vaccine against rhinopneumonia and strangles of horses on laboratory animals.

2. Materials and methods

The new vaccine includes an avirulent strain CB/69 of the equine rhinopneumonia virus, an inactivated strain of Streptococcus equi bacteria "N-5/1" (certificate of deposit of the strain dated 31.05.2018 №1882/11. Registration number VKSHM-B-141P dated 22.05.2018) and the bacterial strain Bacillus subtilis TNP-3 (RF patent for inventions №2703485, publ. 17.10.2019, bul. 29) was used as an immunomodulator. The viral basis of the vaccine was made in the Department of Virology of VIEV (Moscow).

The associated vaccine against rhinopnemonia and horse strangles is a clear liquid from light yellow to yellow-pink in color with a white precipitate, easily broken into a uniform suspension when shaken.

Laboratory tests of an experimental range of the vaccine were carried out on outbred laboratory white mice of both sexes 5-8 weeks old, weighing 18-20 g and healthy male rabbits weighing 1.5-1.6 kg in accordance with the order of the Ministry of Agriculture of the Russian Federation №101 dated March 6, 2018 "On approval of the rules for conducting a preclinical trial of a medicinal product for veterinary use, a clinical trial of a medicinal product for veterinary use, a study of the biequivalence of a medicinal product for veterinary use".

Vials with the drug were viewed in transmitted light to establish foreign impurities, changes in consistency, violations of the integrity and capping of ampoules,

Sterility was determined in accordance with GOST 28085-2013 “Biological medicinal products for veterinary use. Method of bacteriological control of sterility”.

Harmlessness was determined in accordance with GOST 31926-2013 “Medicines for veterinary use. Methods for determining harmlessness”. The experiments were carried out on white mice weighing 18-20 g. The vaccine was injected subcutaneously into the back at a dose of 1.0 cm3 to three white mice. They were observed for 10 days.

The acute toxicity of the associated vaccine against rhinopneumonia and horse strangles was studied on laboratory outbred mice weighing 18-20 g, by observation, weighing, pathomorphological and histological studies after administration of the drug intragastrically on an empty stomach in the morning, at the maximum allowable dose (1 cm3). Prior to the start of the experiments, white mice were observed for 2 days to exclude the weak ones. Formed 2 groups of 3 heads. Mice of the 1st group were injected with the vaccine once, mice of the 2nd group (control group) were injected with a sterile saline solution once. Clinical observations were made within 7 days after drug administration. The studies were carried out in accordance with “Sanitary rules SP 3.3.2.561-96. (approved by the Decree of the State Sanitary and Epidemiological Supervision of the Russian Federation from 31.10.96 №33) 3.3.2. Medical immunobiological preparations”.

The pyrogenicity test was carried out in accordance with GOST 31926-2013 “Medicines for veterinary use. Methods for determining harmlessness”. Pyrogenicity was determined in 2 groups of 3 heads of healthy male rabbits weighing 1.5-1.6 kg. Body temperature was measured in rabbits for three days before testing the drug. The measurement was carried out daily in the morning before giving food using a medical mercury thermometer. The thermometer was administered rectally for 5 minutes.

The determination of the immunogenicity of the viral component of the vaccine was carried out in the VIEV laboratory of virology (Moscow) on outbred mice of 10-14 days of age with a body weight of 6-7 g. Outbred mice were taken from the “Stolbovaya” laboratory animal nursery in the Moscow region. Neurotropic strain “PP1/1” of equine rhinopneumonia virus adapted for growth in mice under the age of 40 days was used in this work [31]. The mice were divided into 2 groups. The 1st group (n=8) received the vaccine twice subcutaneously at 0.3 cm3 with an interval of 14 days. Mice of the 2nd group (n=6) were not vaccinated and were subsequently used as negative and positive controls for experimental infection with the rhinopneumonia virus. Control infection with an adapted strain of equine rhinopneumonia virus at a dose of 0.02 x 6.0 lgTCD50/ml was performed intracerebral in 14 days after revaccination. The effectiveness of immunization was determined by the number of mice resistant to disease and death to infection, and in terms of weight change in comparison with negative (n=3) and positive (n=3) control groups. All experiments with laboratory mice were carried out in accordance with the requirements of biethical standards.

A laboratory test of the immunogenicity of the strangles composition of the associated vaccine against rhinopneumonia and strangles of horses on outbred white mice was carried out in the laboratory of veterinary biotechnology of the Yakut Scientific Research Institute of Agriculture. We used an experimental batch of an associated vaccine against rhinopneumonia and strangles of horses. There were formed 2 groups of experimental animals. A sample of the vaccine mixed from 3 vials was prepared, from which, after shaking, 10 cm3 of the drug was taken into a sterile vial. White mice of group I (10 heads) were vaccinated subcutaneously in the back at a dose of 0.5 cm3. White mice of group 2 were left as controls. Every day after vaccination, a clinical examination of white mice of both groups was performed. For control infection, the average lethal dose of the Streptococcus equi strain "H-5/1" was determined according to the Kerber method modified by I.P. Ashmarin [32]. After 10 days, the mice of the experimental and control groups were infected with a lethal dose Ld50 of strangles streptococcus. There was stablished observation for 10-12 days. The effectiveness of immunization was determined by the number of mice resistant to disease and death.

The research results were mathematically processed according to the Student's method.

3. Results and discussions

Administration of the vaccine preparation intragastrically at a dose of 1 cm3 to experimental white mice and physiological saline at a similar dose to mice in the control group had no effect on the general condition of the animals. The mice completely ate the food, were mobile, and actively showed a lively reaction to others. Thus, signs of intoxication were not found when the associated vaccine against rhinopneumonia and strangles was administered intragastrically in white outbred mice at the maximum allowable dose (1 cm3). Before the start of the experiment, the body weight was determined in the animals of the experimental and control groups, and at the end of the experiment, a control weighing was performed. The dynamics of fluctuations in the body weight of experimental animals as a result of the introduction of the vaccine preparation intragastrically are summarized in fig. 1.

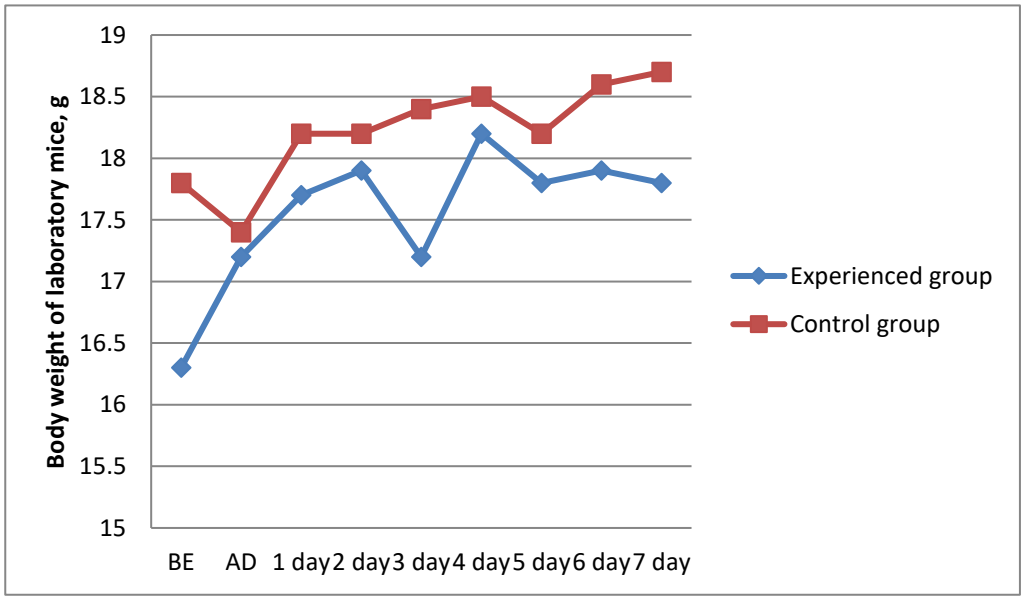

Figure 1. Dynamics of body weight fluctuations in white outbred mice after the administration of an associated vaccine against rhinopneumonia and strangles of horses

«BE» - measurement of body weight before the experiment (within 2 days), «AD» - on the day of the experiment (immediately after the administration of the drug), "1 ... 7" - days of observation.

The average body weight of the animals of the experimental group before the test (during 2 days) was 16.3 ± 9.05 g, and after 7 days after the administration of the drug it was 17.8 ± 10.25 g. Animals of the control group also observed an increase in body weight from 17.8 ± 0.84 g to 18.7 ± 0.63 g. Thus, the analysis of the dynamics of the body weight of white mice revealed its positive increase in both the control and experimental groups. In the experimental group that received the vaccine preparation,

the increase was 9.2%, and in the control group - 5.05%.

Therefore, in mice treated intragastrically with the vaccine preparation, active growth is observed in comparison with the control. The difference was 4.15%. Disease and mortality among vaccinated mice were not noted. Autopsy after necrosis of experimental animals did not show any changes in the internal organs. According to the results of histological studies, no focal dystrophic changes were found in the internal organs (Table 1). Early studies on rats established the absence of toxicity in the probiotic preparation "Sakhabactisubtil" from the bacterial strain Bacillus subtilis TNP-3, from which the immunomodulatory composition of the vaccine was made [33].

Thus, the introduction of an associated inactivated vaccine against rhinopneumonia and strangles based on the avirulent strain CB/69 of the equine rhinopneumonia virus, an inactivated strain of bacteria Streptococcus equi "H-5/1" and an immunomodulator from a strain of bacteria Bacillus subtilis TNP-3, intragastrically to white mice did not have a negative effect on the general condition, contributed to an increase in the average daily gain in live weight of mice, which indicates its harmlessness and safety. The vaccine does not have a toxic, local irritant effect when administered to laboratory white mice.

Table 1. Pathological anatomical autopsy of white mice

Organ	Experimental group	Control group
Light	Dark red-pink staining, swelling	Pattern of oedema
The heart	Brown-red, cavities bloody	No visible macroscopic changes
Liver	Brown-red in colour, not enlarged	No visible macroscopic changes
Spleen	Normal	No visible macroscopic changes
Kidneys	Slightly swollen	No visible macroscopic changes
Stomach, intestines	With air	No visible macroscopic changes

According to the results of a clinical trial, it was also established that the drug in the studied doses does not have an allergenic effect and does not cause convulsive reactions, does not disrupt the coordination of movements in laboratory animals.

The pyrogenic properties of the vaccine were determined on healthy male rabbits in comparison with 0.9% saline. There were formed 2 groups, 3 rabbits in each, which were injected intravenously with samples of the test drug and saline at a dose of 0.1 cm3. The change in body temperature of the animals in the group was determined as the difference between the average temperature measured 30 minutes before the administration of the drug. During observation before the experiment for 3 days, the average temperature of experimental rabbits was 38.6ºC in group 1, and 38.0ºC in group 2. Therefore, before the test, the temperature of the rabbits of the experimental and control groups did not differ significantly. On day 4, temperature was measured twice, the last one 30 minutes before the administration of the drug. The dynamics of fluctuations in the temperature of the animals was recorded every 30 minutes for three hours. On the third hour after the administration of the drug, the temperature of rabbits in group 1 rose by 0.2ºC, and in group 2 by 0.4ºC (table 2). During the clinical examination of the experimental animals of both groups, no changes in the general condition and signs of the disease were recorded. Thus, the studied vaccine preparation was considered non-pyrogenic, since after its administration to rabbits, a slight short-term increase in body temperature was observed in animals of both groups.

Table 2. Measurement of body temperature after drug administration

	Experimental group	Control group
-3 day	38,0±0,21	38,7±0,42
-2 day	37,9±0,21	38,3±0,35
-1 day	38,2±0,28	38,8±0,21
0 day	38,3±0,56	38,5±0,21
-30 min	38,1±0,49	38,5±0,14
Injection of the drug		
+ 30 min	37,6±0,70	38,6±0,21

+ 1 hour	37,9±0,35	38,4±0,42
+ 1.5 hours	38,4±0,28	38,5±0,14
+ 2 hours	38,8±0,14	38,5±0,07
+ 2.5 hours	38,7±0,21	38,7±0,35
+ 3 hours	38,5±0,21	38,7±0,07

Experimental group (3 individuals) - RP vaccine and horse strangles; control group (3 individuals) - 0.9% saline.

The effectiveness of immunization was determined by the number of mice resistant after infection. The immunogenicity of the associated vaccine against the viral component in the first group of mice was 75%, the control mice all died.

In previous laboratory tests on linear mice vaccinated once and twice, the immunogenicity of the inactivated rhinopneumonia vaccine when experimentally infected with VGL-1 was 60%.

For control infection with strangles streptococcus, the average lethal dose (Ld50) was determined according to the Kerber method modified by I.P. Ashmarin [32]. The average lethal dose was LD50=10-3.75 or 200 thousand microbial bodies (with the introduction of 0.2 ml of a diluted suspension).

After 10 days, experimental and control white mice were infected with a lethal dose of Ld50. 2 heads of the ten immunized mice fell ill and died on the second and fifth days of observation. In the control group, all ten white mice fell ill and died. Thus, a sufficiently high immunogenicity of the strangles composition of the vaccine (80%) was established.

The high efficiency of the immunogenic activity of the associated vaccine is due to the high antigenic activity of the CB/69 strain of the rhinopneumonia virus, the bacterial strain Streptococcus equi "H-5/1" and the culture fluid of the bacterial strain Bacillus subtilis TNP-3. It was found that Bac. subtilis TNP-3 has enzymatic, interferon-inducing, antimicrobial, and immunostimulatory properties that enhance the immunogenicity of inactivated vaccines [33]. The cultural liquid (fugate) of the bacterial strain Bacillus subtilis TNP-3 is used as an immunomodulator in the following vaccines: against Salmonella abortion in horses; trivalent vaccine against rhinopneumonia, salmonella abortion and strangles; bivalent vaccine against rhinopneumonia and salmonella abortion; inactivated rhinopneumonia vaccine. These vaccines have shown high immunogenic activity in laboratory and production tests. The results obtained are consistent with the work of researchers who found that viral and bacterial antigens in combined vaccines lead to a significant stimulation of the phagocytic activity of peritoneal macrophages, which contributes to the presentation of these antigens and the development of adaptive immunity [34]. So Liu S.A. and others [35] experimentally proved in laboratory mice that intramuscular administration of a live vector vaccine against GVL-1 stimulates the formation of a humoral and immune response. Kim S.K. and others [36] note that immunization with a weakened strain of the herpesvirus type 1 in laboratory mice stimulates the production of innate immune responses in their bodies and this protects them during control infection with a lethal dose of the virus.

The obtained results of preclinical trials of the vaccine preparation in the future are the basis for conducting a clinical trial of the vaccine on target animals. The development of a bivalent vaccine will increase the effectiveness of preventive measures for respiratory diseases in young horses.

4. Conclusions

Testing of the vaccine on laboratory white mice established its harmlessness. It has been established that when administered orally at the maximum allowable dose, the associated vaccine does not have a pathological effect on the general condition of laboratory white mice. During the next 7 days of observation of the animals, there were no deviations in the condition of the coat and mucous membranes, the nature of the discharge, the dynamics of body weight gain, or death. Pathomorphological and histological studies of the internal organs of experimental animals after the killing of focal dystrophic changes were not revealed. The pyrogenicity test of the vaccine in male rabbits showed it to be non-pyrogenic.

The immunogenicity of the viral composition of the associated vaccine was determined on outbred mice of 10-14 days of age; for control infection there was used the neurotropic strain "PP1/1" of the equine rhinopneumonia virus, adapted to growth in mice. The immunogenicity of the vaccine in the first group of mice was 75%, and the control mice (unvaccinated) all died. The immunogenicity of the strangles composition of the vaccine after control infection with a lethal dose of the bacterial strain Streptococcus equi "H-5/1" LD50 was 80%.

The obtained results of preclinical studies of the associated vaccine against rhinopneumonia and strangles of horses inactivated

with an immunomodulator meet the requirements of vaccine preparations for veterinary use. Preclinical studies have shown that this vaccine has a sufficiently high immunogenic activity and is safe for use in animals. Thus, the efficacy and safety of the associated vaccine against rhinopneumonia and strangles of horses in the laboratory was studied. In the future, based on the results of laboratory tests, production experiments will be carried out to immunize young horses with this vaccine.

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