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Studies on humoral immunity in dogs after use of rabies inactivated vaccines formulated with Montanide ISA 70 VG and GEL 01 adjuvants

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SUMMARY
Despite all the efforts made, the issue of rabies in the world does not lose its relevance. As an acute endemic infection, it poses a considerable danger to both animals and humans. The leading role in the transmission of rabies to humans belongs to diseased dogs; stray animals can be potential sources of rabies agent, which increases the risk of transmitting a deadly virus to humans. Rabies prevention in dogs around the world is carried out by counting the number of these animals, their mandatory routine immunization and evaluating the effectiveness of vaccination against the accumulation of rabies virus-neutralizing antibodies. Inactivated vaccines based on different viral strains formulated with a wide range of adjuvants are used to induce a protective level of humoral immunity against rabies in dogs (≥ 0.5 IU/cm^3), which allows vaccines with high safety and effectiveness for the target animal species to be obtained. The article presents the results of the study of humoral immunity in 20 non-pedigree dogs 21 days after the administration of rabies inactivated vaccines based on culture rabies virus from ARRIAH strain formulated with various adjuvants. The presented rabies vaccines formulated with oil adjuvant Montanide ISA 70 VG and gel adjuvant Montanide GEL 01 were innocuous and safe and induced strong immunity in all vaccinated animals. The vaccine formulated with Montanide ISA 70 VG adjuvant in case of a single administration in the dose of 1.0 cm^3 induces formation of rabies virus-neutralizing antibodies in the level of 2.4 times higher than the vaccine formulated with Montanide GEL 01 adjuvant. The highest levels of rabies antibodies in dogs were 48.1 ± 3.7 and 28.3 ± 1.5 IU/cm^3 and were observed with the use of rabies inactivated emulsion vaccine in the doses of 3.0 and 1.0 cm^3 respectively.

Key words: rabies, inactivated vaccine against rabies, humoral immunity, rabies virus-neutralizing antibodies, dogs.

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Изучение гуморального иммунитета собак при использовании антирабических инактивированных вакцин, изготовленных с применением адъювантов Montanide ISA 70 VG и GEL 01

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РЕЗЮМЕ

Несмотря на все прилагаемые усилия, проблема бешенства в мире не теряет своей актуальности. Являясь острой природно-очаговой инфекцией, представляет огромную опасность как для животных, так и для человека. Ведущая роль в процессе передачи вируса бешенства человеку принадлежит заболевшим собакам, безнадзорные животные могут быть потенциальными источниками возбудителя бешенства, что повышает риск передачи смертельно опасного вируса человеку. Профилактика бешенства среди собак во всем мире осуществляется путем учета численности этого вида животных, их обязательной регулярной иммунизации и оценки эффективности вакцинации по уровню накопления антирабических вируснейтрализующих антител. Для формирования защитного уровня гуморального иммунитета у собак против бешенства (≥ 0,5 МЕ/см^3) применяют инактивированные вакцины, полученные на основе различных штаммов вируса с использованием широкого спектра адъювантов, что позволяет получать вакцины с высокими показателями безопасности и эффективности для целевых видов животных. В статье представлены результаты исследования гуморального иммунитета у 20 беспородных собак на 21 сут после введения антирабических инактивированных вакцин из культурального вируса бешенства штамма «ВНИИЗЖ» с применением различных адъювантов. Представленные вакцины против бешенства, изготовленные с использованием масляного адъюванта Montanide ISA 70 VG и гелевого адъюванта Montanide GEL 01, были авирулентными, безвредными и индуцировали напряженный иммунитет у всех привитых животных. Вакцина на основе адъюванта Montanide ISA 70 VG при однократном введении в дозе 1,0 см^3 способствует выработке вируснейтрализующих антирабических антител в 2,4 раза выше по сравнению с препаратом, полученным с использованием адъюванта Montanide GEL 01. Наиболее высокие титры антител против бешенства у собак составляли 48,1 ± 3,7 и 28,3 ± 1,5 МЕ/см^3 и были отмечены при использовании антирабической инактивированной эмульсионной вакцины в дозах 3,0 и 1,0 см^3 соответственно.

Ключевые слова: бешенство, инактивированная вакцина против бешенства, гуморальный иммунитет, антирабические вируснейтрализующие антитела, собаки.

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INTRODUCTION

Rabies is a major viral disease of humans and animals and also one of the most dangerous zoonoses, causing lesions of the central nervous system, encephalomyelitis, and paralysis with inevitable lethal outcome. The agent belongs to the order *Mononegavirales*, family *Rhabdoviridae*, genus *Lyssavirus,* species *Rabies lyssavirus* [1].

Rabies is a world-wide problem, to which international organizations (WHO, OIE, FAO, GARC) and veterinary services of many countries pay special attention [2, 3]. The disease leads to significant costs associated with animal mortality, management of outbreak consequences, preventive and quarantine measures, management of wild animal population, catching of stray cats and dogs and diagnostic testing [4, 5]. It is estimated that the annual global economic loss from rabies is more than $8.6 billion [6].

The virus spreads easily, so the disease can become an epizootic. A large number of natural foci of rabies is mainly maintained due to wild animals, though the pathogen is also transmitted to domestic animals, and this fact requires increased attention because of their close proximity to humans [7]. It is considered that the leading role in the process of rabies virus transmission to humans belongs to diseased dogs because of their high sensitivity to the virus as well as a number of biological and ecological features peculiar to them (tendency to form packs, ability to move considerable distances, etc.). Most cases of rabies transmission from dogs to humans are associated with viral saliva getting into wounds inflicted by bites [4, 8]. Currently, more than half of the population of the Russian Federation has companion animals. At the same time, the problem of free-roaming dogs is acute, and their packs have become common in cities, towns and settlements [7, 9]. They may be potential sources of the rabies agent, which increases the risk of transmitting the deadly virus to humans.

Rabies prevention in dogs worldwide is carried out by taking into account the number of this animal species, its mandatory routine immunization, and by assessing the effectiveness of vaccination against the level of accumulation of rabies virus-neutralizing antibodies (VNA). To induce a protective level of humoral immunity in dogs against rabies (≥ 0.5 ME/cm^3) [2], inactivated vaccines obtained from different rabies virus strains and formulated with a wide range of adjuvants are used, which allows to obtain vaccines with high safety and efficiency indicators for target animal species [2, 5, 9, 10, 11].

The aim of this work was to study the possibility of protecting dogs from rabies through the use of rabies inactivated vaccines based on "ARRIAH" strain of rabies virus and formulated with Montanide ISA 70 VG and GEL 01 adjuvants.

MATERIALS AND METHODS

Vaccines. Experimental batches of rabies inactivated vaccines were made based on "ARRIAH" rabies virus strain, reproduced in suspension cell line from the Syrian baby hamster kidney (BHK-21). Rabies virus was inactivated with aminoethylethylenimine (AEEI) solution. The suspension of the inactivated antigen was purified from ballast proteins by simple sedimentation. To enhance immune response, vaccines were formulated with Montanide ISA 70 VG oil adjuvant, consisting of mineral oil and non-ionic emulsifier, and Montanide GEL 01 gel adjuvant, based on highly purified finely dispersed sodium polyacrylate (SEPPIC, France) (Table 1).

Laboratory animals. We used thirty non-pedigree dogs weighing 10-15 kg, and twenty white mice weighing 10-12 g.

All tests on animals were conducted in strict accordance with the Interstate Standards for the keeping and care of laboratory animals GOST 33216-2014 and GOST 33215-2014, adopted by the Interstate Council for Standardization, Metrology and Certification, as well as the requirements of Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes.

Immunization of animals. For the purposes of the research, all dogs were divided into 6 groups per 5 animals, the vaccines were administered by intramuscular route. The following animals were immunized with rabies inactivated adsorbed vaccine: animals of group 1 (No. 1–5) were immunized with a single undiluted dose of 1.0 cm^3, animals of group 2 (No. 6–10) – at a dose of 3.0 cm^3, animals of group 3 (No. 11–15) – twice at a dose of 1.0 cm^3 with inoculation interval of 7 days. Rabies emulsion vaccine was administered to dogs of group 4 (No. 16–20) a single time at a dose of 1.0 cm^3, group 5 (No. 21–25) – at a dose of 3.0 cm^3, group 6 (No. 26–30) – twice at a dose of 1.0 cm^3 with an interval between inoculations of 7 days (Table 2).

Evaluation of innocuity of rabies virus antigen in cell culture. The innocuity of the inactivated rabies virus suspension was controlled by inoculation into the monolayer continuous BHK-21 cell line during three consecutive passages using rabies immunoglobulin G labeled with FITC. As a positive control, we used a freeze-dried suspension of non-inactivated rabies virus of "ARRIAH" strain with infectivity titer of 7.00 lg TCID50/cm^3. A freeze-dried suspension of inactivated rabies virus served as negative control. The material was considered avirulent if no fluorescent glow specific to rabies virus was detected in any of the three consecutive passages [2].

Evaluation of safety of rabies inactivated vaccines on white mice. Before administration to white mice, the emulsion vaccine against rabies was destroyed up to the isolation of the antigenic phase. For this purpose, the preparation was triply frozen and thawed, and then centrifuged for 30 min at 7000 g (rotor diameter – 6 cm, speed – 10,000 rpm). The process resulted in the sedimentation of the antigen at the bottom of the tube, which was used for testing by intracerebral injection of 0.03 cm^3 to ten white mice.

The adsorbed vaccine was diluted 4-fold by the saline solution and the resulting suspension was injected intracerebrally per 0.03 cm^3 to ten mice [2].

The animals were observed for 21 days. The vaccine was considered safe if all vaccinated animals remained clinically healthy during the whole period of observation, without rabies signs [2].

Testing of safety of the vaccine on dogs. For the analysis of the vaccines for safety, ten animals from groups 2 and 5 (No. 6–10 – for adsorbed vaccine, No. 21–25 – for emulsion vaccine) were administered a triple dose of the vaccine into neck muscles (3.0 cm^3 each). The safety of the re-administration of a single dose of these vaccines into neck muscles at a 7-day interval was also assessed in 10 dogs of groups 3 and 6 (No. 11–15 – for adsorbed vaccine, No. 26–30 – for emulsion vaccine). The clinical condition of the animals was observed for 14 days. The vaccine was considered safe on condition that all dogs remained clinically healthy at the end of the observation period, without necrosis in the inoculation area.

Table 1
Rabies inactivated vaccines for dogs made of ARRIAH strain of rabies virus formulated with different adjuvants

Таблица 1
Антирабические инактивированные вакцины для собак, изготовленные на основе штамма «ВНИИЗЖ» вируса бешенства с применением разных адъювантов

No.	Type of vaccine	Adjuvant	Adjuvant/ antigen ratio	Type of emulsion
1	inactivated adsorbed	Montanide GEL 01	10/90	–
2	inactivated emulsion	Montanide ISA 70	70/30	W/O

W/O – water-in-oil (inverse emulsion).

Determination of the number of rabies virus-neutralizing antibodies (VNA). Evaluation of strength of post-vaccination humoral rabies immunity in dogs 21 days after vaccination was carried out by analysis of sera taken from animals No. 1–5, 16–30 in neutralization reaction (FAVN modification) with the use of monolayer cell line BHK-21 according to the OIE recommendations on rabies [2]. Each serum was tested in triplicate.

Statistical data processing consisted in determination of arithmetic mean values of rabies antibody titer and reliability of statistical difference between the mean values according to Student-Fisher method [12].

RESULTS AND DISCUSSION

At the first stage of the study the innocuity of the obtained rabies virus antigen of "ARRIAH" strain in the monolayer cell line BHK-21 was evaluated. As a result of the analysis, no fluorescent glow specific to rabies virus was detected in any of the three consecutive passages, which indicated the complete inactivation of the virus.

At the second stage, two proposed vaccines were tested for innocuity in 20 white mice and safety in 20 dogs (Nos. 6–15, 21–30). According to the results of the analysis, the produced vaccines were found to be innocuous and safe, since all immunized animals remained clinically healthy

Table 2
Immunization of dogs with rabies inactivated vaccines based on ARRIAH strain of rabies virus formulated with different adjuvants

Таблица 2
Иммунизация собак антирабическими инактивированными вакцинами на основе штамма «ВНИИЗЖ» вируса бешенства и разных адъювантов

Group No.	Animal No.	Vaccine characteristics		Inoculation dose, cm^3
		type	adjuvant	
1	1–5	inactivated adsorbed	Montanide GEL 01	1.0
2	6–10			3.0
3	11–15			1.0 + 1.0
4	16–20	inactivated emulsion	Montanide ISA 70 VG	1.0
5	21–25			3.0
6	26–30			1.0 + 1.0

Table 3
Evaluation of level of post-vaccination humoral immunity against rabies in dogs by FAVN after administration of rabies inactivated vaccines formulated with different adjuvants ($n_{of\ tests} = 3, p < 0.005$)

Таблица 3
Оценка степени поствакцинального гуморального иммунитета у собак против бешенства в FAVN после введения антирабических инактивированных вакцин с применением разных адъювантов ($n_{исследований} = 3, p < 0,005$)

Group No.	Vaccine characteristics		Inoculation dose, cm^3	Animal No.	Titer of rabies VNA 21 days after vaccination, IU/cm^3
	type	adjuvant			
1	inactivated adsorbed	Montanide GEL 01	1.0	1	13.4 ± 1.2
				2	13.5 ± 0.8
				3	10.3 ± 0.9
				4	10.9 ± 1.5
				5	10.9 ± 1.7
				M ± m	**11.8 ± 1.5**
4	inactivated emulsion	Montanide ISA 70 VG	1.0	16	29.6 ± 1.7
				17	28.6 ± 1.4
				18	27.4 ± 1.5
				19	29.8 ± 1.5
				20	26.3 ± 1.6
				M ± m	**28.3 ± 1.5**
5	inactivated emulsion	Montanide ISA 70 VG	3.0	21	43.4 ± 2.8
				22	52.3 ± 3.9
				23	50.8 ± 3.6
				24	48.5 ± 3.7
				25	45.6 ± 3.9
				M ± m	**48.1 ± 3.7**
6	inactivated emulsion	Montanide ISA 70 VG	1.0 + 1.0	26	17.5 ± 0.8
				27	15.5 ± 1.5
				28	14.9 ± 1.4
				29	17.2 ± 0.8
				30	16.9 ± 0.8
				M ± m	**16.4 ± 1.1**

during the whole period of observation, without signs of rabies and without tissue necrosis at the injection site.

The next stage of the study was devoted to studying post-vaccination humoral immunity in 20 dogs after administration of rabies vaccines based on "ARRIAH" strain of rabies virus and formulated with Montanide ISA 70 VG and Montanide GEL 01 adjuvants. The animals of four groups were immunized according to the scheme presented in Table 2. Before and 21 days after vaccination, blood was taken from dogs and serums were tested by neutralization test (FAVN modification) [2]. It was found that prior to vaccination, the serum did not contain antibodies against rabies virus.

Data in Table 3 and Figures 1–4 show that VNA titers in the animals of group 1 vaccinated with rabies inactivated adsorbed vaccine formulated with Montanide GEL 01 adjuvant once at a dose of 1.0 cm^3, averaged 11.8 ± 1.5 ME/cm^3. This value is 2.4 times lower than that of group 4 where dogs were immunized once at a dose of 1.0 cm^3 with Montanide ISA 70 VG adjuvant and the antibodies against rabies virus were 28.3 ± 1.5 IU/cm^3. Given that the level of humoral immunity in dogs vaccinated with the emulsion vaccine is higher than in the case of the adsorbed vaccine, further studies were conducted with a vaccine formulated with Montanide ISA 70 VG adjuvant, and the dose was increased three times, and the animals were vaccinated twice at a dose of 1.0 cm^3 at 7-day intervals. Thus, in animals of group 5 which were administered the emulsion vaccine once at a dose of 3.0 cm^3, VNA titers stood at 48.1 ± 3.7 ME/cm^3, which was 4.1 and 2.4 times higher than in animals of groups 1 and 4 respectively. VNA titers in dogs of group 6 which were vaccinated with this preparation twice per 1.0 cm^3, were on average 16.4 ± 1.1 IU/cm^3, which was 1.4 times higher than in animals of group 1.

As a result of the comparative analysis of the obtained data, it was established that the rabies inactivated emulsion vaccine formulated with Montanide ISA 70 VG adjuvant stimulated the formation of a stronger humoral immunity in comparison with the adsorbed vaccine formulated with Montanide GEL 01 adjuvant. Thus, in the group of animals immunized with the emulsion vaccine a single time at a dose of 1.0 cm^3, the VNA titer was 1.7 times higher compared to the data for adsorbed vaccine. The level of rabies antibodies in dogs immunized with emulsion vaccine once at a dose of 3.0 cm^3 and twice at a dose of 1.0 cm^3 was 4.1 and 1.4 times higher respectively compared to animals vaccinated with the adsorbed vaccine.

Comparing average values of rabies antibodies titers in dogs 21 days after vaccination with emulsion vaccine at different doses, it was found that the highest level of VNA was achieved with a single administration of the preparation at a dose of 3.0 cm^3 (48.1 ± 3.7 IU/cm^3).

A single administration of the rabies vaccine to dogs at a dose of 1.0 cm^3 and a double administration at a dose of 1.0 cm^3 21 days after immunization induced accumulation of VNA in titers of 28.3 ± 1.5 and 16.4 ± 1.1 IU/cm^3, which is 2.4 and 2.9 times lower in comparison with a single administration of the above-mentioned vaccine at a dose of 3.0 cm^3. It should be noted that during the whole period of observation (21 days) the condition of all animals vaccinated with the presented vaccines at different doses was satisfactory.

As a result of the conducted studies, it was found that the developed rabies inactivated vaccines based on "ARRIAH" strain of rabies virus formulated with Montanide ISA 70 VG and Montanide GEL 01 adjuvants 21 days after the administration induced protective level of antibodies in dogs (above 0.5 IU/cm^3) and thus met the OIE requirements for immunogenicity [2]. At the same time the highest levels of humoral immunity in dogs against rabies of 48.1 ± 3.7 and 28.3 ± 1.5 IU/cm^3 were observed when using rabies inactivated emulsion vaccine at doses of 3.0 and 1.0 cm^3 respectively.

CONCLUSION

The immunobiological properties of two rabies inactivated vaccines based on "ARRIAH" strain of rabies

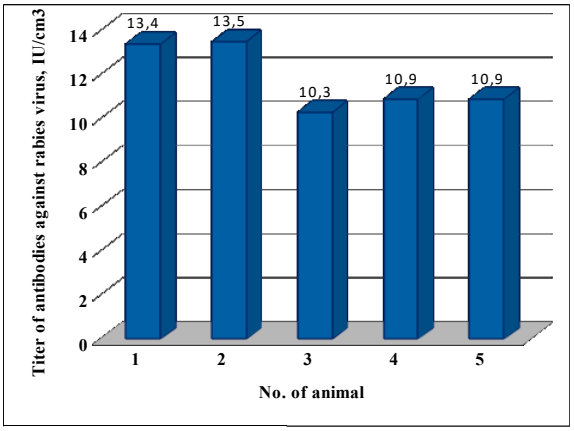

Fig. 1. Humoral immunity in dogs 21 days after single administration of anti-rabies inactivated adsorbed vaccine based on Montanide GEL 01 adjuvant at a dose of 1.0 cm³ (according to the FAVN data)

Рис. 1. Гуморальный иммунитет у собак на 21 сут после однократного введения антирабической инактивированной сорбированной вакцины на основе адъюванта Montanide GEL 01 в дозе 1,0 см³ (по данным FAVN)

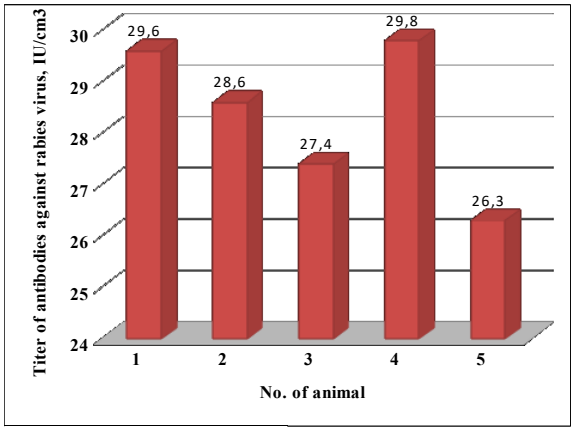

Fig. 2. Humoral immunity in dogs 21 days after single administration of anti-rabies inactivated emulsion vaccine based on Montanide ISA 70 VG adjuvant at a dose of 1.0 cm³ (according to the FAVN data)

Рис. 2. Гуморальный иммунитет у собак на 21 сут после однократного введения антирабической инактивированной эмульсионной вакцины на основе адъюванта Montanide ISA 70 VG в дозе 1,0 см³ (по данным FAVN)

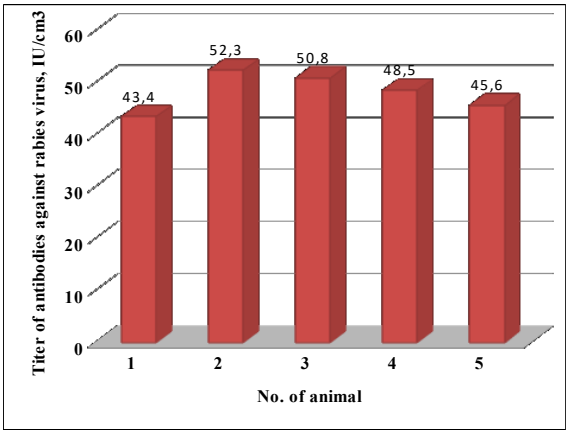

Fig. 3. Humoral immunity in dogs 21 days after single administration of anti-rabies inactivated emulsion vaccine based on Montanide ISA 70 VG adjuvant at a dose of 3.0 cm³ (according to the FAVN data)

Рис. 3. Гуморальный иммунитет у собак на 21 сут после однократного введения антирабической инактивированной эмульсионной вакцины на основе адъюванта Montanide ISA 70 VG в дозе 3,0 см³ (по данным FAVN)

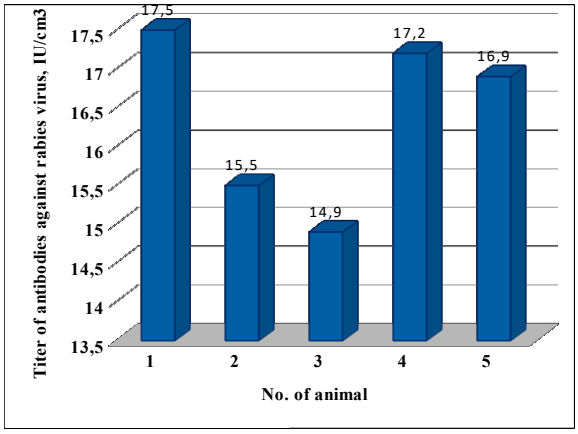

Fig. 4. Humoral immunity in dogs 21 days after double administration of anti-rabies inactivated emulsion vaccine based on Montanide ISA 70 VG adjuvant at a dose of 1.0 cm³ at 7-day interval (according to the FAVN data)

Рис. 4. Гуморальный иммунитет у собак на 21 сут после двукратного введения антирабической инактивированной эмульсионной вакцины с применением адъюванта Montanide ISA 70 VG по 1,0 см³ с интервалом 7 сут (по данным FAVN)

virus were studied using Montanide ISA 70 VG and Montanide GEL 01 adjuvants.

When tested on white mice and dogs, it was found that the obtained vaccines were safe.

Humoral immunity was assessed in 20 non-pedigree dogs 21 days after the administration of the developed vaccines at different doses. It was found that the developed rabies inactivated vaccines stimulated the formation of protective level of antibodies in dogs and met the OIE requirements for immunogenicity.

It was found that the highest levels of humoral immunity in dogs against rabies 21 days post-vaccination were 48.1 ± 3.7 and 28.3 ± 1.5 IU/cm³ and were observed when using rabies inactivated emulsion vaccine at doses of 3.0 and 1.0 cm³ respectively. The vaccine formulated with Montanide ISA 70 VG oil adjuvant at a single administration of a dose of 1.0 cm³ contributed to the formation of rabies VNA at the level 2.4 times higher compared to the vaccine formulated with Montanide GEL 01 adjuvant.

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