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Possible outcomes of reassortment *in vivo* between wild type and live attenuated influenza vaccine strains

Irina Kiseleva*, Irina Dubrovina, Ekaterina Bazhenova, Ekaterina Fedorova, Natalie Larionova, Larisa Rudenko

Institute of Experimental Medicine, RAMS, St. Petersburg, Russia

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ABSTRACT

Reassortment of influenza viruses in nature has been well documented. Genetic reassortment plays a key role in emergence of new influenza A strains, including pandemic viruses. Permissive host can be simultaneously coinfected with multiple influenza viruses. During genetic reassortment gene segments are exchanged between parental viruses that may lead to some enhancement of virulence of reassortant progeny. At present, vaccination with live attenuated cold-adapted (*ca*) reassortant vaccine (LAIV) is used as an effective public health measure for influenza prophylaxis. However, there are concerns about a potential of simultaneous infection of human host with *ca* and wild type (*wt*) influenza viruses which might produce progeny that contain novel, more virulent genotypes. The aim of this study was to investigate potential consequences of reassortment of *wt* with LAIV strains *in vivo*.

We demonstrated that reassortment of *wt* viruses with *ca* strains in guinea pigs have resulted in progeny virus which caused reduced macroscopic lesions of chicken embryos. According to phenotypical data 95% (19 out of 20) isolated reassortants were restricted in replication at elevated temperature of 40 °C. None of reassortants were more virulent than *wt* parents, or revealed significantly higher macroscopic lesions than *wt* parental viruses. Our results suggest that genetic reassortment between *wt* and vaccine strain is unlikely to lead to virulent reassortant progeny. These findings provide additional support of LAIV safety data.

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1. Introduction

Influenza is a severe viral infection that affects people regardless of age and remains a major health problem worldwide [6]. Influenza illness is accompanied by high mortality, especially in young children and the elderly. Influenza epidemics occur each year and affect up to 15% of the population. Pandemics occur every 10–40 years. Three major properties of the influenza virus determine its pandemic potential: the novelty of a strain to the immune system, virulence and ability to spread from person to person (transmissibility) [1].

In spite of the important role of the transmissibility of the influenza virus, a little is known about the nature and its mechanisms. Understanding the mechanisms that underlie transmissibility will allow more effective monitoring of influenza and to explore new ways and methods of its prevention. The most effective current method of protection against influenza is vaccination. In recent years, cold-adapted live attenuated influenza vaccine (*ca* LAIV) as a means of protection against influenza has consolidated its position in the public health arena among other preventive measures [7,8]. However, the question is periodically raised about the possibility of the spread of live influenza vaccine strains in the population and their subsequent reassortment with circulating seasonal or pandemic viruses. Reassortment of influenza viruses is a widespread natural phenomenon and genetic reassortment plays a key role in the emergence of new strains of influenza, including pandemic influenza [9,10]. In mixed infections segments of the genome can be exchanged between the parental viruses, which could lead to the emergence of reassortants with enhanced virulence. Some believe that the vaccine strain might exchange genes with the seasonal virus that may lead to a mutant virus with new unexplored properties and increased virulence [2]. Thereby there are fears that widespread use of LAIV could increase the potential risk of transmission of vaccine strain or its reassortment with circulating influenza viruses resulting in generation of reassortants of enhanced pathogenicity. The proof that these fears are unfounded will confirm the safety of use of live influenza vaccine not only in epidemic, but in the pandemic influenza period also.

The aim of this research was to study potential consequences of reassortment of wild type influenza viruses with LAIV.

* Corresponding author at: Institute of Experimental Medicine, 12 Acad. Pavlov Street, 197376 St. Petersburg, Russia. Tel.: +7 812 234 6860; fax: +7 812 234 9214.
E-mail address: irina.v.kiseleva@mail.ru (I. Kiseleva).

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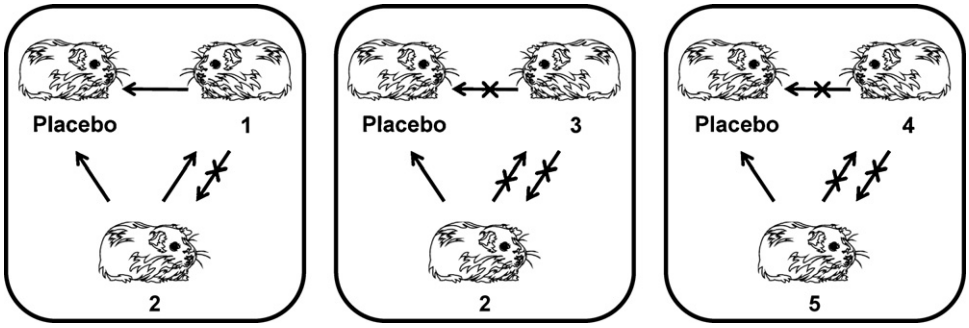

Fig. 1. Respiratory droplet transmission among co-caged guinea pigs: 1, A/California/07/2009 (H1N1) influenza pandemic virus; 2, NIBRG-23 (H5N1), PR8-based vaccine strain for inactivated prepandemic influenza vaccine which inherited HA and NA from A/turkey/Turkey/1/2005 (H5N1); 3, A/17/Leningrad/134/17/57 (H2N2), MDV for Russian LAIV; 4, A/17/California/2009/38 (H1N1), Russian pLAIV; 5, A/Sydney/5/97 (H3N2) seasonal influenza virus.

2. Materials and methods

2.1. Viruses

Wild type influenza viruses – A/Sydney/5/97 (H3N2) and A/California/07/2009 (H1N1) – were obtained from CDC (Atlanta, GA, USA). NIBRG-23 (H5N1)-PR8-based vaccine strain for inactivated prepandemic influenza vaccine which inherited HA and NA from A/turkey/Turkey/1/2005 (H5N1) – was obtained from the World Health Organization (WHO). The A/Leningrad/134/17/57 (H2N2) is a cold-adapted master donor virus (MDV) for Russian reassortant live attenuated influenza vaccines (LAIV). A/17/California/2009/38 (H1N1) is a pandemic LAIV strain (pLAIV) generated by classical reassortment of A/California/07/2009 (H1N1) with the MDV in embryonated hen's eggs. Viruses were propagated in 10–11-day-old embryonated hen's eggs at the temperature of 32 °C.

2.2. Macroscopy of chicken embryos

Macroscopic lesions were scored as follows: 0, no visible changes; 1, mild; 2, moderate; 3, strong; 4, severe.

2.3. Animals

Female guinea pigs weighing 300–350 g were obtained from Laboratory Animal Farm "RAPPOLOVO" (Rappolovo, North-West region, Russia). Animals were allowed free access to food and water. All animals were seronegative to tested viruses on arrival. Guinea pigs were housed in standard rat cages at relative humidity 25% and ambient temperature of 22 °C. Animals were inoculated with influenza viruses intranasally with a total of 6.0 log EID_{50} in a volume of 0.4 ml (0.2 ml per nostril) without anesthesia. Nasal washes and lung samples were collected at 3 dpi and cloned by limited dilutions in the presence of anti-parental viruses' sera in 10–11-day-old embryonated eggs.

Transmissibility of influenza virus was determined by its presence in samples of collected specimens incubated in hen's eggs and by hemagglutination inhibition (HAI) test at 28 dpi.

2.4. Hemagglutination inhibition (HAI) test

Guinea pig serum samples were treated with receptor-destroying enzyme (RDE) (Denka-Seiken, Tokyo, Japan) and then were tested for hemagglutination-inhibition specific antibodies by standard procedure [3] using 1% suspension of human 0(I) Rh+ red blood cells.

2.5. Determining ts phenotype

Capacity of influenza viruses to grow at optimum (32 °C) and elevated (38 °C and 40 °C) temperatures was determined by titration in eggs and expressed as a reduction of virus titer at 40 °C (38 °C) from the titer at permissive temperature (32 °C), respectively. The log EID_{50}/ml calculation was based on the Reed–Muench method [4]. Viruses were considered as *non-ts* if (log EID_{50}/ml at 32 °C) - (log EID_{50}/ml at 40 (38) °C) $\leq$ 3.0–3.5 log EID_{50}/ml. The cold-adapted viruses (A/Leningrad/134/17/57 MDV and A/17/California/2009/38 pandemic LAIV) were used as positive controls of *ts* marker.

2.6. RNA isolation

RNA was isolated from influenza virus infected egg's allantoic fluid by using "RIBO-sorb" (AmpliSens, Russia).

2.7. Genome composition of reassortants

Genome composition of reassortant influenza A viruses was monitored by RT-PCR followed by restriction fragment length polymorphism (RFLP) analysis as described [5].

3. Results

3.1. Transmission of influenza viruses in guinea pigs

Animals were infected with the A/California/07/2009 (H1N1) pandemic influenza virus, A/Sydney/5/97 (H3N2) seasonal influenza virus, NIBRG-23 (H5N1) PR8-based reassortant influenza virus or *ca* viruses – A/Leningrad/134/17/57 (H2N2) MDV and A/17/California/2009/38 (H1N1) pLAIV. The animals of the placebo group were mock-inoculated intranasally with PBS. The transmission of influenza viruses between co-caged infected or infected and non-infected (placebo) guinea pigs was studied. The results show that A/California/07/2009 (H1N1), A/Sydney/5/97 (H3N2) and NIBRG-23 (H5N1) viruses are contagious agents which can be transmitted between co-caged naïve guinea pigs (Fig. 1). In addition, it was demonstrated that when animals inoculated with A/California/07/2009 were co-caged with those inoculated with NIBRG-23, they got infected with both viruses. Thus, influenza virus transmission from H5N1- to H1N1-infected pigs has been shown but not other way around (Fig. 1, left panel).

Cold-adapted (*ca*) viruses were not transmitting between guinea pigs (Fig. 1, middle and right panels). But theoretical possibility of reassortment of LAIV with wild type (*wt*) viruses followed by their transmission exists. Thus, the other objective of this study was to assess the probability of potential reassortment of

cold-adapted viruses with *wt* strains resulting in reassortants. To demonstrate outcomes of possible reassortment *in vivo* between wild type and vaccine strains, animals were co-infected with *wt* and *ca* viruses by intranasal administration of a mixture of two viruses.

3.2. Guinea pig-derived reassortants of NIBRG-23 (H5N1) with MDV (H2N2)

Animals were infected with a mixture of NIBRG-23 and MDV. At 3 dpi nasal washes were collected and resulting viruses cloned by limited dilution in the presence of anti-NIBRG-23 or anti-MDV immune serum. Nine reassortants were isolated and their *ts* phenotype was assessed compared to *ts* phenotype of parental viruses. Phenotype of parental viruses was different - MDV was shown to be *ts* both at 40 °C and at 38 °C in contrast to NIBRG-23, which was *non-ts* at 38 °C. The *ts* phenotype of all reassortants was typical of the MDV-reassortants were temperature sensitive both at 40 °C and 38 °C (Table 1).

3.3. Guinea pig-derived reassortants of A/Sydney/5/97 (H3N2) and A/17/California/2009/38 (H1N1) pLAIV

Simultaneous infection is more or less artificial. In reality, individuals vaccinated with LAIV would more likely be infected with *wt* virus shortly before or after vaccination. To mimic this theoretical situation, guinea pigs were inoculated with A/17/California/2009/38 (H1N1) pLAIV and A/Sydney/5/97 (H3N2) *wt* virus simultaneously or vaccinated with pLAIV 24 h prior or post infection with *wt* virus. At 3 dpi nasal washes and lung tissue samples were collected and influenza viruses cloned by limited dilution in the presence of anti-A/California/07/2009 (H1N1) or anti-A/Sydney/5/97 (H3N2) immune serum. In the group pre-vaccinated with pLAIV, virus was detected in nasal washes but not in lungs. After simultaneous administration virus was found both in nasal washes and in lung tissue. Vaccination 24 h after inoculation of A/Sydney/5/97 (H3N2) did not lead to virus in the samples at 3 dpi. Eleven reassortants were isolated (six from nasal washes and five from lungs) and their *ts* phenotype was assessed compared to *ts* phenotype of parental viruses.

A/17/California/2009/38 (H1N1) pLAIV was *ts* both at 40 °C and at 38 °C in contrast to A/Sydney/5/97 (H3N2), which was very *non-ts* effectively replicating at 40 °C.

All reassortants except one (reassortant #16 which was derived from lungs) displayed particular temperature sensitive phenotype because they lost the ability to grow at 40 °C. Thus, in general, reassortants were less attenuated then pLAIV but more attenuated then *wt* parent (Table 2).

3.4. Genome composition of guinea pig-derived reassortants

In total, 20 reassortants were isolated. Genome composition analysis showed that 18 out of 20 reassortants inherited PB2 from MDV, 9 out of 20 reassortants inherited PB1 from MDV and 5 out of 20 reassortants inherited PA gene from MDV. It was noted that, 19 reassortants out of 20 possessed at least one polymerase gene from MDV and only one lung derived reassortant #16 inherited polymerase complex from A/Sydney/5/97 (H3N2) *wt* virus. Overall, 57 genes belonged to MDV and 103 genes belonged to different *wt* viruses.

3.5. Macroscopy observations

Embryonated chicken eggs were infected with 5.0 log EID_{50}/ml of tested viruses. After 48 h of incubation at 32 °C macroscopy lessions of embryos were investigated. Infection of chicken embryos

Table 1
Generation of triple reassortants of NIBRG-23 (H5N1) with A/Leningrad/134/17/57 (H2N2) MDV in guinea pigs.

Viruses (reassortants)	Genes								Mean log reduction (*ts* phenotype) of virus titer[a] (log EID_{50}/ml) at:		Macroscopy embryo lesions score[b] (average)[c]
	PB2	PB1	PA	HA	NP	NA	M	NS	32 °C/40 °C	32 °C/38 °C	
Parental viruses											
NIBRG-23 (H5N1)[d]	PR8[e]	PR8	PR8	Tur[f]	PR8	Tur	PR8	PR8	6.2 (*ts*)	0 (*non-ts*)	4, 4, 4, 4, 3 (3.8)
A/Leningrad/134/17/57 (H2N2) MDV	L17[g]	L17	L17	L17	L17	L17	L17	L17	7.0 (*ts*)	6.0 (*ts*)	1, 0, 0, 0, 0 (0.2)
Reassortants generated *in vivo* (simultaneous administration of NIBRG-23 and MDV)											
1 (nasal wash derived reassortant)	L17	PR8	PR8	Tur	PR8	L17	PR8	PR8	7.5 (*ts*)	7.5 (*ts*)	1, 0, 2, 0, 1 (0.8)
2 (nasal wash derived reassortant)	L17	PR8	PR8	Tur	PR8	L17	PR8	PR8	7.6 (*ts*)	7.6 (*ts*)	1, 1, 1, 0, 0 (0.6)
3 (nasal wash derived reassortant)	L17	PR8	PR8	Tur	PR8	L17	PR8	PR8	7.5 (*ts*)	7.5 (*ts*)	1, 0, 1, 0, 0 (0.4)
4 (nasal wash derived reassortant)	L17	L17	PR8	Tur	PR8	L17	PR8	L17	6.0 (*ts*)	6.0 (*ts*)	1, 0, 1, 0, 0 (0.4)
5 (nasal wash derived reassortant)	L17	L17	PR8	Tur	PR8	L17	PR8	L17	6.1 (*ts*)	6.1 (*ts*)	1, 0, 1, 1, 0 (0.6)
6 (nasal wash derived reassortant)	L17	L17	PR8	Tur	PR8	L17	PR8	L17	6.2 (*ts*)	6.2 (*ts*)	1, 0, 1, 1, 0 (0.6)
7 (nasal wash derived reassortant)	L17	L17	PR8	Tur	PR8	L17	PR8	L17	6.0 (*ts*)	6.0 (*ts*)	1, 0, 1, 0, 0 (0.4)
8 (nasal wash derived reassortant)	L17	L17	PR8	Tur	L17	L17	L17	L17	6.7 (*ts*)	6.7 (*ts*)	1, 1, 1, 0, 1 (0.8)
9 (nasal wash derived reassortant)	L17	L17	PR8	Tur	L17	L17	L17	L17	6.1 (*ts*)	6.1 (*ts*)	1, 0, 1, 0, 0 (0.4)

[a] From the titer at permissive temperature (32 °C).
[b] Macroscopy lesions score: 0, no visible changes; 1, mild; 2, moderate; 3, strong; 4, severe.
[c] Average score per group. Number of chicken embryos in each group – 5.
[d] Reassortant of A/turkey/Turkey/1/2005 (H5N1) with A/PR/8/34 (H1N1).
[e] Gene belongs to A/PR/8/34 (H1N1) influenza virus.
[f] Gene belongs to A/turkey/Turkey/1/2005 (H5N1) avian influenza virus.
[g] Gene belongs to A/Leningrad/134/17/57 (H2N2) master donor virus.

Table 2
Generation of double and triple reassortants of A/Sydney/5/97 (H3N2) seasonal virus with A/17/California/2009/38 (H1N1) pLAIV in guinea pigs.

Viruses (reassortants)	Genes								Mean log reduction (*ts* phenotype) of virus titer[a] ($\log EID_{50}$/ml) at:		Macroscopy embryo lesions score[b] (average)[c]
	PB2	PB1	PA	HA	NP	NA	M	NS	32 °C/40 °C	32 °C/38 °C	
Parental viruses											
A/California/07/2009 (H1N1) *wt*	Cal[d]	Cal	Cal	Cal	Cal	Cal	Cal	Cal	1.2 (*non-ts*)	0.5 (*non-ts*)	4, 4, 2, 4, 4, 3 (3.5)
A/Sydney/5/97 (H3N2) *wt*	Syd[e]	Syd	Syd	Syd	Syd	Syd	Syd	Syd	1.3 (*non-ts*)	1.0 (*non-ts*)	3, 4, 2, 4, 3, 3 (3.2)
A/Leningrad/134/17/57 (H2N2) MDV	L17[f]	L17	L17	L17	L17	L17	L17	L17	7.0 (*ts*)	6.0 (*ts*)	1, 0, 0, 0, 0, 0 (0.2)
A/17/California/2009/38 (H1N1) pLAIV	L17	L17	L17	Cal	L17	Cal	L17	L17	8.0 (*ts*)	7.0 (ts)	0, 0, 0, 1, 0, 0 (0.2)
Reassortants generated *in vivo* (administration of pLAIV was performed 24 h prior infection with A/Sydney/5/97)											
10 (nasal wash derived reassortant)	L17	Syd	L17	Syd	Syd	Syd	L17	Syd	6.0 (*ts*)	1.3 (*non-ts*)	1, 1, 0, 1, 1, 0 (0.7)
11 (nasal wash derived reassortant)	L17	Syd	L17	Syd	Syd	Syd	L17	Syd	6.7 (*ts*)	1.0 (*non-ts*)	1, 0, 1, 1, 0, 0 (0.5)
12 (nasal wash derived reassortant)	L17	L17	Syd	Syd	L17	Syd	Syd	Syd	5.5 (*ts*)	0.8 (*non-ts*)	1, 0, 1, 0, 1, 0 (0.5)
13 (nasal wash derived reassortant)	L17	L17	Syd	Syd	L17	Syd	Syd	Syd	5.3 (*ts*)	1.9 (*non-ts*)	1, 0, 0, 0, 1, 1 (0.5)
Reassortants generated *in vivo* (simultaneous administration of pLAIV and A/Sydney/5/97)											
14 (nasal wash derived reassortant)	L17	Syd	Syd	Syd	L17	Syd	Syd	Syd	6.0 (*ts*)	0.3 (*non-ts*)	1, 1, 0, 1,1, 0 (0.7)
15 (nasal wash derived reassortant)	L17	Syd	Syd	Syd	Syd	Syd	Syd	Syd	6.0 (*ts*)	0 (*non-ts*)	0, 1, 1, 0, 1, 0 (0.5)
16 (lung derived reassortant)	Syd	Syd	Syd	Cal	Syd	Syd	L17	Syd	3.5 (*non-ts*)	1.8 (*non-ts*)	1, 2, 1, 1, 2, 1 (1.3)
17 (lung derived reassortant)	L17	Syd	L17	Cal	Syd	Syd	Syd	Syd	7.2 (*ts*)	1.7 (*non-ts*)	1, 1, 1, 0, 1, 0 (0.7)
18 (lung derived reassortant)	L17	Syd	L17	Cal	Syd	Syd	Syd	Syd	6.4 (*ts*)	0.2 (*non-ts*)	1, 0, 0, 1, 1, 1 (0.7)
19 (lung derived reassortant)	L17	Syd	L17	Cal	Syd	Syd	Syd	Syd	7.8 (*ts*)	1.8 (*non-ts*)	1, 0, 1, 1, 0, 1 (0.7)
20 (lung derived reassortant)	Syd	L17	Syd	Cal	L17	Syd	Syd	Syd	6.0 (*ts*)	0.7 (*non-ts*)	1, 0, 0, 1, 1, 1 (0.7)

[a] From the titer at permissive temperature (32 °C).
[b] Macroscopy lesions score: 0, no visible changes; 1, mild; 2, moderate; 3, strong; 4, severe.
[c] Average score per group. Number of chicken embryos in each group – 6.
[d] Gene belongs to A/California/07/2009 (H1N1) pandemic influenza virus.
[e] Gene belongs to A/Sydney/5/97 (H3N2) seasonal influenza.
[f] Gene belongs to A/Leningrad/134/17/57 (H2N2) master donor virus.

with a H1N1 2009 virus caused significant pathological changes (average score = 3.5). The macroscopy of chicken embryos infected with avian H5N1-PR8 reassortants demonstrated that they also were severely affected (average score = 3.8). The most frequently observed macroscopic lesions included focal head and skin hemorrhages, head edema, delayed embryo development, weight loss, kinked neck, and skin loss. Most dominant gross lesions were observed in embryo head while body was less affected. In contrast, the mock (PBS) (average score = 0), MDV (average score = 0.2), and pLAIV (average score = 0.2) infected chicken embryos did not show any significant macroscopic lesions (Tables 1 and 2). Macroscopy scores in groups of guinea pig derived reassortants did not dramatically differ from the animals infected with cold-adapted viruses. Visualization results revealed that all reassortants when inoculated in eggs did not induce substantial macroscopic lesions and were not more virulent than *wt* parents. Nineteen reassortants out of 20 (##1–15 and 17–20) had average score from 0.4 to 0.8 (Tables 1 and 2). The only *non-ts* reassortant #16 did show some visible macroscopy lesions (from mild to moderate) but they were not as severe as lesions induced by its *wt* parental virus A/Sydney/5/97 (H3N2) (average score was 1.3 and 3.2, respectively).

4. Discussion

In this research natural transmission of influenza viruses between co-caged guinea pigs was studied. A/California/07/2009 (H1N1) pandemic virus and A/Sydney/5/97 (H3N2) seasonal virus were effectively transmitted under conditions where direct contact between infected and naïve animals occurred. Co-caging of naïve guinea pigs with animals inoculated with *wt* and *ca* viruses demonstrated that the only one transmission route exists – from animals infected with *wt* virus to naïve animals. Viruses did not transmit in any of the other directions (from *wt* virus to *ca* virus, from *ca* virus to *wt* virus or placebo).

NIBRG-23 (H5N1) virus was very transmissible despite the fact that its hemagglutinin gene was modified to decrease its pathogenicity (multibasic cleavage site associated with high virulence was deleted). Influenza virus transmission from H5N1- to *wt* H1N1-infected guinea pigs has been observed.

Thus, our research did not demonstrate natural transmission of cold-adapted viruses, thereby supporting LAIV safety claims. Although there is the theoretical possibility of reassortment of *ca* virus with wild type virus, phenotypical and macroscopic analysis of reassortants derived after forced co-infection of guinea pigs with *ca* and *wt* viruses demonstrated that all of them were attenuated or, at least, less virulent than their *wt* parental viruses. None of *in vivo* derived reassortants acquired enhanced pathogenicity.

Influenza virus hemagglutinins differ with regard to their specificity for host cell receptors. Recent data indicate that receptor specificity may affect the transmissibility of influenza viruses [11]. However, transmissibility may be associated with some other factors as well because it was demonstrated that live cold-adapted reassortant vaccine strains which inherited hemagglutinin from transmissible wild type viruses did not transmit among contact animals.

5. Conclusion

We believe that our data provide additional strong support for the safety of LAIV and indicate that indeed the risk of reassortment in nature between cold-adapted and wild type viruses is lower than expected but more in-depth studies are needed to fully confirm this.

Conflict of interest statement

The authors declare that they have no conflicts of interests.

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