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Rapid Communication

Neutralizing antibodies to human and simian adenoviruses in humans and New-World monkeys

Jonatan Ersching [a], Malva I.M. Hernandez [b], Fabrizzio S. Cezarotto [c], Jovino D.S. Ferreira [d], Amely B. Martins [e], William M. Switzer [g], Zhiquan Xiang [f], Hildegund C.J. Ertl [f], Carlos R. Zanetti [a], Aguinaldo R. Pinto [a,*]

[a] Departamento de Microbiologia, Imunologia e Parasitologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, 88040-900, Florianópolis, SC, Brazil
[b] Departamento de Ecologia e Zoologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, 88040-900, Florianópolis, SC, Brazil
[c] Centro de Hematologia e Hemoterapia do Acre, Av. Getúlio Vargas 2787, 69914-500, Rio Branco, AC, Brazil
[d] Hospital Universitário Polydoro Ernani de São Thiago, Universidade Federal de Santa Catarina, 88040-900, Florianópolis, SC, Brazil
[e] Centro Nacional de Pesquisa e Conservação de Primatas Brasileiros, Praça Antenor Navarro 5, 58010-480, João Pessoa, PB, Brazil
[f] The Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104, USA
[g] Centers for Disease Control and Prevention, 1600 Clifton Rd., Atlanta, GA 30333, USA

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ABSTRACT

Vaccines based on adenovirus (Ad) vectors are currently in development against several pathogens. However, neutralizing antibodies (NAb) to human adenovirus type 5 (AdHu5), the best-studied vector, are highly prevalent in humans worldwide. Less-prevalent adenoviruses, including human and simian serotypes, provide alternative vaccine platforms. In this study, sera from 200 Brazilian human subjects and New-World monkeys were tested for NAb titers to human serotypes AdHu5 and AdHu26 and chimpanzee-origin Ad viruses of serotype 6 (AdC6) and serotype 68 (AdC68). Seroprevalence rates of NAb in humans were 69.5% for AdHu5, 44% for AdHu26, 21% for AdC6 and 23.5% for AdC68. In addition, NAb titers to human Ad were consistently higher than those found to simian serotypes. Surprisingly, sera from some New-World monkey species were able to neutralize AdC6 and/or AdC68. A possible explanation for these findings and the implications for the development of Ad-vector vaccines are discussed in detail.

Introduction

Adenoviruses (Ad), belonging to the *Adenoviridae* family, are nonenveloped DNA viruses that have been studied as vaccine vectors as they are highly immunogenic, stable, safe and easy to manipulate (Tatsis and Ertl, 2004). Recombinant Ad vectors elicit potent humoral and cellular immunity, especially $CD8^+$T cells, against their transgene products. Ad vectors are currently considered as vaccine candidates against several intracellular pathogens, including HIV (Buchbinder et al., 2008), *Plasmodium falciparum* (Shott et al., 2008), *Trypanosoma cruzi* (de Alencar et al., 2009) and *Mycobacterium tuberculosis* (Mu et al., 2009).

Human adenovirus serotype 5 (AdHu5) is the best-studied serotype. However, humans are commonly infected with AdHu5 early in life and consequently develop specific neutralizing antibodies (NAb) in childhood (Appaiahgari et al., 2007; Thorner et al., 2006). By neutralizing the vector, antibodies dampen the transgene product-specific immune responses elicited by recombinant AdHu5-based vaccines, as was shown in studies with mice (Barouch et al., 2004), rhesus monkeys (Casimiro et al., 2003) and humans (Catanzaro et al., 2006). These findings may hamper clinical use of AdHu5 vectors as vaccine carriers since seroprevalence of NAb to AdHu5 is very high worldwide, particularly in the developing world (Dudareva et al., 2009; Mast et al., 2010; Pilankatta et al., 2010), where vaccines to pathogens such as HIV or *Plasmodium* are most direly needed.

To overcome this drawback, rare adenovirus serotypes can be employed as substitutes for or in combination with AdHu5 in vaccine strategies. Pre-clinical studies demonstrated that, among rare human adenovirus serotypes, the human adenovirus type 26 (AdHu26) is one of the most immunogenic vectors, even though it is not as immunogenic as AdHu5 (Abbink et al., 2007). Moreover, AdHu26 is currently been employed in two Phase-I clinical trials as an HIV-1 vaccine vector candidate (ClinicalTrials, 2010). Another source of rare adenovirus serotypes includes those from other species, such as chimpanzees. In particular, the chimpanzee adenovirus serotypes AdC6 and AdC68 figure among promising candidates as vaccine carriers (revised in Tatsis and Ertl, 2004). Both AdC viruses, which are from two distinct serotypes related to the human adenovirus serotype 4, are able to grow in HEK293 complementing cells and are not cross-neutralized by antibodies against common human adenovirus serotypes (Farina et al., 2001). In addition, we have demonstrated in pre-clinical studies that AdC6 and AdC68 vectors are highly

* Corresponding author. Fax: +55 48 3721 9258.
E-mail address: pintoar@ccb.ufsc.br (A.R. Pinto).

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immunogenic and effective at inducing cytotoxic T lymphocytes specific to HIV-1 antigens (Pinto et al., 2004; Reyes-Sandoval et al., 2004, Souza et al., 2007).

Nonetheless, studies comparing the occurrence of NAb to AdHu5, AdHu26, AdC6 and AdC68 are limited and mainly available for North America, Europe and Africa (Mast et al., 2010; Xiang et al., 2006). These reports suggest considerable differences in NAb prevalence according to geographic region. Prevalence rates in Latin America are poorly known, with only one study reporting NAb to human adenoviruses in Brazil (Mast et al., 2010). Because these populations may become recipients of Ad-based vaccines, it is pivotal to assess pre-immunity to Ad-vector candidates. Therefore, the aim of the present study was to determine NAb titers to AdHu5, as well as to its possible substitutes AdHu26, AdC6 and AdC68 in Brazil. In order to better understand the origin of NAbs to AdC viruses found in humans, samples from non-human primates were also evaluated.

Results

Neutralizing antibodies to adenoviral vectors in humans

NAb titers to AdHu5, AdHu26, AdC6 and AdC68 were assessed by a virus neutralization assay with GFP-expressing recombinant Ad vectors. Antibodies against AdHu5 and AdHu26 were found in 69.5% and 44% of human samples, respectively (Table 1). Unexpectedly, 21% and 23.5% of Brazilian subjects also featured NAb to chimpanzee AdC6 and AdC68 serotypes, respectively. The seroprevalence rate to AdHu5 was statistically higher than to AdHu26 ($P=0.0067$, χ^2 test), AdC6, and AdC68 ($P<0.0001$ in both cases, χ^2 test). Therefore, among our Brazilian study population, pre-existing immunity is more common to AdHu5 than to AdHu26, AdC6 and AdC68. In accordance to the seroprevalence findings, Brazilians also featured higher NAb titers to AdHu5 than to AdHu26, AdC6 or AdC68 ($P<0.0001$, Friedman ANOVA test). As shown in Fig. 1, NAb titers to AdHu5 were found at a wider range and the median titer (320) was higher than those to the other vectors (20 for AdHu26 and 10 for AdC6 and AdC68). Accordingly, 53% of the Brazilian subjects had high (>200) NAb titers to AdHu5, and 22% exhibited titers above 1000, whereas only 9% of Brazilians had NAb titers >200 to AdHu26 (Fig. 2). Titers were lower to AdC6 and AdC68, only 2.5% and 2.0% of human samples showed NAb titers >200 to these viruses, respectively (Fig. 2). Interestingly, NAb titers >1000 were not found to AdHu26, AdC6 or AdC68. Taken together, these findings indicate that antibody responses against AdHu5 are stronger or more sustained than those to the other viruses, suggesting that the use of Ad vectors other than AdHu5 may be considered as vaccine carriers in Brazil.

Neutralizing antibodies to adenoviral vectors in New-World monkeys

The unexpected occurrence of NAb to simian Ad among Brazilian adults at rates higher than previously reported in the US or Thailand gave rise to the hypothesis that chimpanzee adenoviruses may circulate among monkeys from Brazil, notwithstanding that chimpanzees are Old-World primates and are typically only found in zoos in Brazil. Therefore, samples from two monkey species that are commonly in contact with Brazilians, common marmosets (*Callithrix jacchus*) and capuchin monkeys (*Cebus libidinosus*), were also analysed for NAb. Both monkey species were either free-ranging or kept as pets. Neutralizing antibodies to AdHu5, AdC6 and AdC68, but not to AdHu26, were found in sera from common marmosets, as shown in Table 1. In this species, NAb to AdC6 and AdC68 were more frequent than to AdHu5 ($P<0.02$ in both cases, χ^2 test). Additionally, NAb titers to AdC6 and AdC68 were higher than those observed to AdHu5 ($P<0.0001$, Friedman ANOVA test) (Fig. 1). Nonetheless, NAb titers >200 among common marmosets were rare and found only for AdC6 (Fig. 2). In contrast, sera from capuchin monkeys were not able to neutralize AdHu5, AdHu26, AdC6 or AdC68 (Table 1), except for one specimen which featured NAb titers 640 to AdHu5 and AdC68, and 320 to AdC6 (Fig. 1). We had previously tested sera from chimpanzees obtained from zoos of the United States for antibodies to the AdC viruses and found such antibodies in all of the animals (Xiang et al., 2006). To assess if antibodies to AdC viruses were common in New-World monkeys, we tested sera obtained from an additional 84 animals belonging to a variety of species and living in primate centers and zoos in the United States for NAbs to AdHu5, AdHu26 and AdC6. None of the animals were positive for AdHu5 or AdHu26 virus

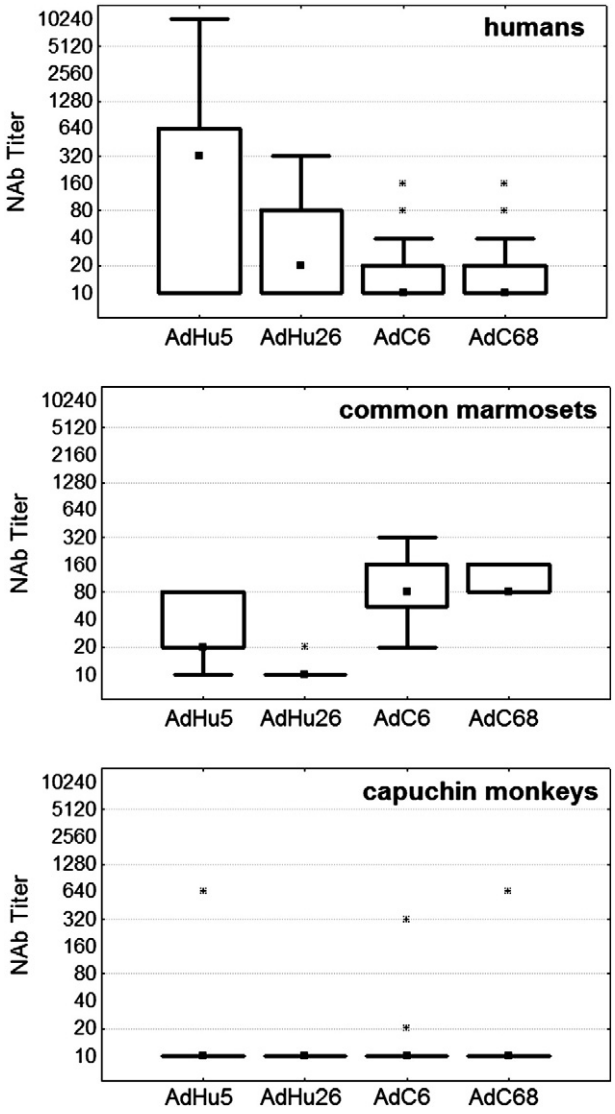

Fig. 1. Neutralizing antibody titers to human (AdHu5 and AdHu26) and chimpanzee (AdC6 and AdC68) adenoviruses in humans, common marmosets (*Callithrix jacchus*) and capuchin monkeys (*Cebus libidinosus*) from Brazil. Symbols: square = median, rectangle = interquartile range, whisker = non-outlier range, and asterisk = outlier.

Table 1
Seroprevalence of neutralizing antibodies to human (AdHu5 and AdHu26) and simian (AdC6 and AdC68) adenoviruses in Brazil.

Species		n	Positive samples[a] (%)			
			AdHu5	AdHu26	AdC6	AdC68
Human	(*Homo sapiens*)	200	69.5	44.0	21.0	23.5
Common marmoset	(*Callithrix jacchus*)	16	31.3	0.0	93.8	100.0
Capuchin monkey	(*Cebus libidinosus*)	18	5.5	0.0	5.5	5.5

[a] Samples with NAb titers higher than 20 were scored as positive.

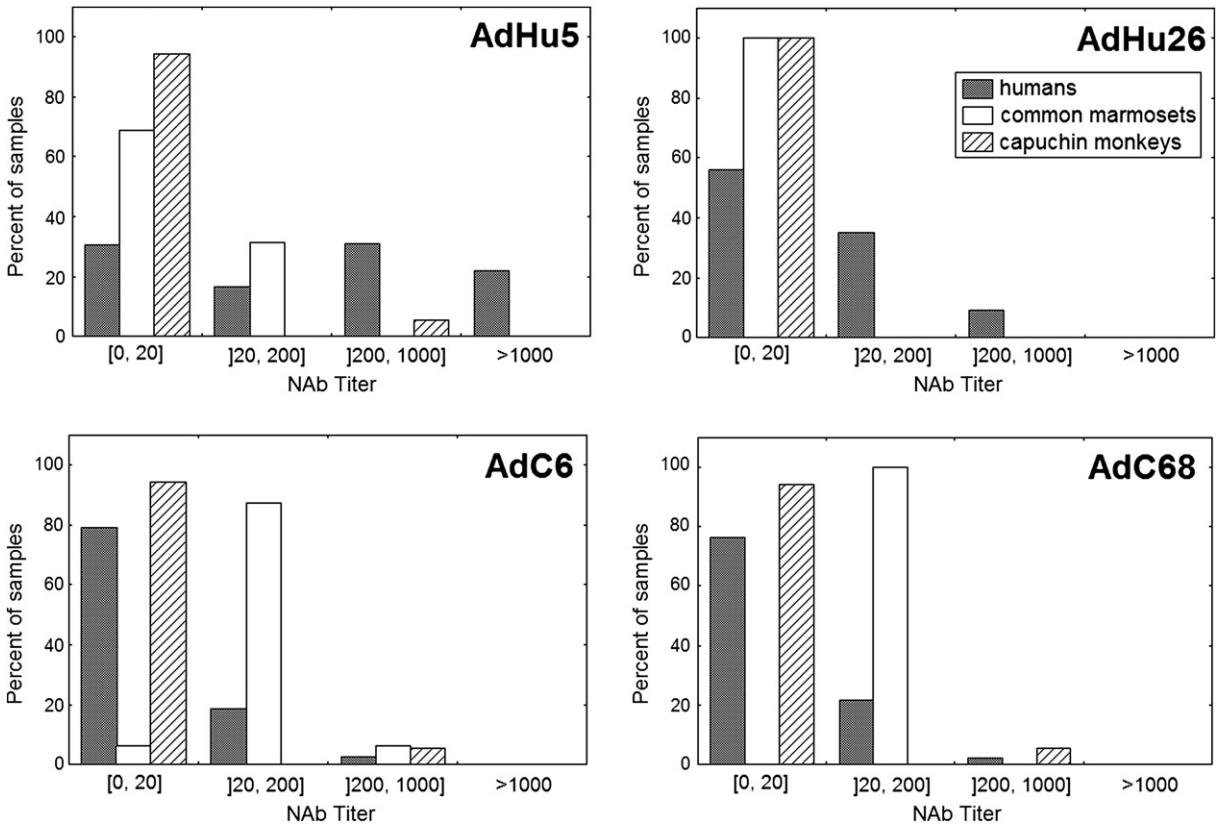

Fig. 2. Distribution of serum samples from humans, common marmosets (*Callithrix jacchus*) and capuchin monkeys (*Cebus libidinosus*) from Brazil according to the NAb titer to human (AdHu5 and AdHu26) and chimpanzee (AdC6 and AdC68) adenoviruses.

(Table 2), only one tamarin monkey (*Saguinus oedipus*) tested positive for AdC6 at a titer of 20, three additional tamarins (1 *S. oedipus* and 2 *Saguinus mystax*) scored at a 10 titer for NAbs to AdC6. In addition we tested sera from 39 rhesus macaques obtained from a primate facility, which also houses chimpanzees, for antibodies to AdC6 to assess if the AdC viruses readily spread between different primate species. As can be seen in Table 2, 12.5% of rhesus macaques had antibodies to AdC6; 4 had low titers of 20 and only one animal had titer of 80.

Table 2
Neutralizing antibody titers to AdC6 among New and Old-World monkeys from the United States.

Species		n	NAb titer to AdC6[a] (%)				
			Undetectable	10	20	40	80
Capuchin[b]	(*Cebus apella*)	23	100	0	0	0	0
Capuchin[b]	(*Cebus albifrons*)	1	100	0	0	0	0
Marmoset[b]	(*Callithrix jacchus*)	5	100	0	0	0	0
Geoffrey's marmoset[b]	(*Callithrix geoffroyi*)	5	100	0	0	0	0
Owl monkey[b]	(*Aotus trivirgatus*)	4	100	0	0	0	0
White-faced Saki monkey[b]	(*Pithecia pithecia*)	7	100	0	0	0	0
Black-handed spider monkey[b]	(*Ateles geoffroyi*)	8	100	0	0	0	0
Brown-handed spider monkey[b]	(*Ateles fusciceps*)	3	100	0	0	0	0
Squirrel monkey[b]	(*Samiri bolivinis*)	4	100	0	0	0	0
Squirrel monkey[b]	(*Saimiri boloviensis peruviensia*)	3	100	0	0	0	0
Tamarin[b]	(*Sagiunus mystax*)	12	83	17	0	0	0
Tamarin[b]	(*Sagiunus oedipus*)	9	78	11	11	0	0
Rhesus macaque[c]	(*Macaca mullata*)	39	87.5	0	10	0	2.5

[a] NAb titers to AdHu5 and AdHu26 were undetectable in all animals tested.
[b] New-World monkey.
[c] Old-World monkey.

Discussion

Prior studies have reported a 60% NAb prevalence to AdHu5 in Europe (Kostense et al., 2004; Mast et al., 2010), 35–70% in North America (Nwanegbo et al., 2004; Sumida et al., 2005) 75–100% in Asia (Pilankatta et al., 2010; Xiang et al., 2006) and 60–100% in Africa (Abbink et al., 2007; Dudareva et al., 2009; Xiang et al., 2006). Here we report 70% AdHu5 NAb prevalence among Brazilians, thus reinforcing that these antibodies are frequent in humans throughout the world. These findings are consistent with a recent study indicating 80% NAb seroprevalence to AdHu5 in Brazil (Mast et al., 2010). Neutralizing antibodies to AdHu5 are commonly present in human sera at high titers, especially in developing countries. In Subsaharan Africa, 48% of human samples featured NAb to AdHu5 at titers >1000 (Abbink et al., 2007). In the present study, 22% of Brazilian subjects harbored NAb titers >1000 to AdHu5. Because high-titer NAb to Ad reduce the effectiveness of vaccines based on the homologous Ad serotype (Casimiro et al., 2003), these data discourage the clinical use of AdHu5-based vectors as vaccine carriers.

Differently, AdHu26 seems to be less prevalent than AdHu5, reaching NAb prevalence rates of 12% in North America, 60% in Asia, 20–90% in Africa (Abbink et al., 2007; Mast et al., 2010; Thorner et al., 2006) and, as shown here, 44% in Brazil. Notably, NAb titers to AdHu26 are considerably lower than to AdHu5. In this study, while 53% of human samples featured NAb titers to AdHu5 >200, 9% harbored NAb to AdHu26 at these titers. AdHu26 has been shown to be more immunogenic than other rare human Ad serotypes and might be useful as a surrogate vaccine candidate for AdHu5 in countries with low seroprevalence rates (Abbink et al., 2007). Notwithstanding, it remains to be investigated at what titers NAb affect transgene product-specific immune responses to AdHu26 vectors, which are not as immunogenic as AdHu5 vectors.

As demonstrated in the present report, NAb titers to AdC viruses were significantly lower than to human Ad, and less than 3% of Brazilian subjects featured NAb at titers >200 to AdC. Considering the problem of NAb pre-existence, in addition to the pre-clinical studies that provide evidence of the high immunogenicity of AdC6 and AdC68, our findings indicate that chimpanzee adenoviruses may be better vaccine vector candidates than human Ad.

The AdC viruses evaluated in this study are described as chimpanzee-specific viruses (Tatsis and Ertl, 2004), reaching seroprevalence rates of 90% in the species *Pan troglodytes* (Farina et al., 2001; Xiang et al., 2006). This is the first report to describe NAb to AdC viruses in Latin America, reporting seroprevalence rates of approximately 20% in humans in Brazil for either AdC6 or AdC68. Neutralizing antibodies against AdC have been reported to occur in lower prevalence rates in North America (2–4%), and even in chimpanzee-endemic regions, such as Southeast Asia (1.5–3%) and Africa (2–20%) (Xiang et al., 2006). The causative aspects for these findings among our Brazilian study population are difficult to be addressed, especially because chimpanzees are not endemic in the Americas. It is reasonable to suppose that AdC viruses might be cross-neutralized by antibodies against human Ad viruses. However, both AdC6 and AdC68 belong to subgenera E of *Adenoviridae* and AdHu4, the only human serotype of this group, does not show serological cross-reactivity with AdC68 (Xiang et al., 2002). Nonetheless, it is also possible that human antibodies specific to other pathogens endemic in Brazil could cross-react with AdC viruses.

Alternatively, Ad viruses serologically related to AdC viruses may circulate in other primate species, including New-World monkeys, and the relatively high seroprevalence rates of NAb to AdC viruses in Brazilians might be caused by spillover infections from monkeys to humans. The latter possibility is further supported by our finding that some of the New-World monkeys residing in Brazil had antibodies that neutralized AdC viruses while such antibodies were not detected in New-World monkeys residing in the USA. In fact, NAb to AdC6 and AdC68 were found in sera from 93.8 and 100% of common marmosets from Brazil, respectively, which clearly corroborates this hypothesis. Adenoviruses have co-evolved with their hosts for more than 400 million years (Benkö and Harrach, 2003). Therefore, due to their phylogenetic proximity, it is possible that humans and monkeys have already been infected with similar adenoviruses. Human Ad viruses have been isolated from Old-World monkeys in China, and zoonotic transmissions to humans have also been suggested (Roy et al., 2009). Furthermore, as shown here, NAb to AdC viruses could be detected at low levels in some sera from rhesus macaques living in captivity at a facility where they were likely to have contact with chimpanzee feces, which commonly contain infectious Ad viruses (Roy et al., 2009).

We also observed absence of NAb to Ad in capuchin monkeys from Brazil. These findings are in agreement with an extensive study of sera from a variety of New-World monkeys kept at primate centers and zoos in the United States, which failed to show antibodies to AdHu5 and AdHu26 virus. Additionally, none of the marmosets from the USA had antibodies to AdC6 virus and only one tamarin, which as marmosets belong to the *Callitrichinae* family, had only low NAb titers to AdC6. Therefore, the occurrence of NAb to AdC6, AdC68 and AdHu5 among common marmosets from Brazil is puzzling.

Differences in prevalence of NAb to Ad in sera from common marmosets from Brazil or USA facilities are curious and may reflect the more controlled environment of the latter. It is also valuable of note that the common marmosets from Brazil included in this study were from a restricted population from a remnant vegetation area within the city of Salvador (BA). Since these free-ranging animals were located within an urban region, they might have been in contact with humans and may have contracted AdHu5 virus from them. In addition, there is a zoo that harbors chimpanzees in Salvador. Therefore, a possible explanation to our results is that the common marmosets were somehow in contact with chimpanzees, or chimpanzee feces, and were infected by AdC serotypes. A second possibility is that the common marmosets from Brazil harbor pathogens such as simian adenoviruses that induce antibodies capable of cross-neutralization of AdC viruses. The specificity of such antibodies, however, remains elusive and future studies are necessary to clarify this important issue.

In conclusion, the present report shows that NAb to AdHu5 and AdHu26 are commonly found in humans from Brazil, and seroprevalence rates of NAb to AdC6 and AdC68 are comparatively rare. Unexpectedly, a few individuals, as well as common marmosets residing in Brazil have NAb titers to AdC6 and AdC68, which may indicate that these serotypes circulate in New-World monkeys in Brazil and may thus not be chimpanzee viruses.

Materials and methods

Cells

HEK293 cells (American Type Culture Collection CRL-1573), a human embryonic kidney cell line that expresses E-1 gene from AdHu5, were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Cultilab) supplemented with 10% heat-inactivated fetal calf serum (FCS; LGC), 100 UI/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B (all from Sigma-Aldrich). Cells were cultured to confluency at 37 °C and 5% CO_2, washed with PBS, trypsinized, suspended in DMEM supplemented with 10% FCS, and manually counted after trypan blue staining.

Recombinant adenoviruses

Replication-defective, GFP-expressing Ad vectors from serotypes AdHu5, AdHu26, AdC6 and AdC68 were constructed in the Wistar Institute's Vector Core Facility (Philadelphia, PA) based on a previously described protocol (Davis et al., 2001). The recombinant vectors were serially passaged on complementing HEK293 cells to generate high-titer viral stocks. Adenovirus vectors were purified by cesium chloride gradient centrifugation, dialyzed, diluted in PBS to a final concentration of 5×10^{12} viral particles/mL, and stored at −80 °C.

Human serum samples

Human serum samples were obtained from 200 healthy Brazilian blood donors aged between 18 and 65. One hundred subjects were recruited at Centro de Hematologia e Hemoterapia do Acre/HEMOACRE (Rio Branco, AC), and one hundred subjects were recruited at Hospital Universitário Polydoro Ernani de São Thiago (Florianópolis, SC). At each site of enrollment, 50 male and 50 female subjects were recruited. From each subject, 5 mL of peripheral blood was collected in plastic tubes with clot activator and gel for serum separation. Tubes were centrifuged at 1000 ×*g* for 15 min, and serum samples were stored at −20 °C. Before use, sera were heat-inactivated at 56 °C for 30 min. Ethical approval was obtained from the Ethics Committee of Universidade Federal de Santa Catarina, and participants provided informed consent.

Non-human primate serum samples

Samples from captive and wild monkeys from Northeast Brazil were included in the study. Sera from 16 common marmosets (*C. jacchus*) and 18 capuchin monkeys (*C. libidinosus*) were provided by Centro Nacional de Pesquisa e Conservação de Primatas Brasileiros (João Pessoa, PB). In addition, 84 samples from other species of *Atelidae*, *Pithecelidae* and *Cebidae* monkeys and 39 samples from rhesus macaques were obtained from primate centers and zoos in the United States. Samples were aliquoted and stored at −20 °C.

Adenovirus neutralization assays

Ad neutralization assays were performed as described previously (Sprangers et al., 2003), with some modifications. The ratio of viral particles per cell and the post-infection incubation period were calibrated to optimize assay sensitivity. Briefly, 1×10^7 viral particles of GFP-expressing AdHu5, AdHu26, AdC6 or AdC68 were mixed with twofold serial dilutions (ranging from 1:10 to 1:10,240) of heat-inactivated sera and incubated for 1 h at 37 °C and 5% CO_2 in 96-well plates. After incubation, 2×10^4 HEK 293 cells were added per well. Sera from BALB/c mice primed and boosted with 1×10^{10} viral particles of GFP-expressing vectors were used as positive controls. As negative control, DMEM was added instead of serum samples. Following a 24 h incubation at 37 °C and 5% CO_2, cells were fixed with paraformaldehyde 4% and nuclei staining was performed with Hoechst 5 μg/mL. Plates were analysed under an epifluorescence microscope (BX41, Olympus) in 40× objective lense and fields were photographed. NAb titers corresponded to the reciprocal dilutions in which the ratio of GFP-expressing/Hoechst-stained cells reached approximately 50% of the ratio observed in negative controls. Titers higher than 20 were scored as positive for the presence of serotype-specific NAb. For each Ad serotype, results are representative of two or more independent experiments.

Data analysis

Statistical analyses were performed with Satistica 7 software. Comparisons between seroprevalence rates of AdHu5 and the other serotypes were made using chi-square (χ^2) test. In order to compare neutralizing antibody titers among the four adenoviral serotypes, Friedman ANOVA test was applied. In all tests, P values<0.05 were assumed as significant.

Conflict of interest

The authors declare no conflict of interest. HCJE has patents pending for the use of AdC viruses as vaccines.

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