Recombinant adeno-associated viruses (AAVs) have unique gene-transfer properties that speak to their potential as carriers for gene therapy or vaccine applications. However, the presence of neutralizing antibodies to AAV as a result of previous exposure can significantly limit effective gene transfer. In this study, we obtained 888 human serum samples from healthy volunteers in 10 countries around the world. Samples were assayed for neutralizing antibodies to AAV1, AAV2, AAV7, and AAV8, as well as to a novel, structurally distinct AAV vector, rh32.33, in an in vitro transduction inhibition assay. Our data revealed that neutralizing antibodies to AAV2 were the most prevalent antibodies in all regions, followed by antibodies to AAV1. The seroprevalences of antibodies to AAV7 and to AAV8 were lower than that for antibodies to AAV1, and neutralization of AAVrh32.33 was only rarely detected. Our data also indicate a strong linkage of seroreactivity between apparently distinct serotypes that has not been predicted previously in animal models.

The adeno-associated virus (AAV) serves as a promising gene delivery system because of its safety profile, its ability to transduce both dividing and nondividing cells, and its proven record of efficacy. AAV has been detected in many different human tissues [1–3] but has not been associated with any disease. Preexisting immunity to AAV can limit effective gene transfer [4, 5]. Animal studies indicate this limitation is most likely to occur through antibody-mediated neutralization of the incoming vector particles in a serotype-specific manner [5–8].

In the early 1970s, several groups reported frequencies of antibodies to AAV1 and AAV2 ranging from 30% to 80% among humans [9–13]. Since then, several other AAV serotypes and >100 natural AAV variants have been isolated from tissue specimens of humans and nonhuman primates [14–16]. In preclinical models, the AAV7 and AAV8 serotypes have, in recent years, emerged as interesting candidates for gene therapies. Studies using polyclonal rabbit antiserum have demonstrated only a low level of cross-reactivity between AAV1, AAV2, AAV7, and AAV8 in terms of the repertoire of humoral responses they elicit [17]. In addition, because AAV7 and AAV8 were isolated from nonhuman primates, antibodies to these viruses were anticipated to have lower seroprevalences among humans. These data were consistent with the use of AAV7 and AAV8 as vectors in human populations that have high frequencies of seroreactivity toward AAV2 and AAV1. Analysis of human IgG (intravenous immunoglobulin [IVIG]) pooled from large groups of individuals revealed that AAV8 was 10 times more resistant to neutralization than AAV2 [18], yet to date there is limited information available on the seroprevalence of antibodies toward AAV7 and AAV8 in humans.

The goal of this study was to determine the frequencies of AAV7 and AAV8 specific and cross-reactive neutralizing antibodies (NAbs) among humans and compare them with the frequencies of AAV1 and AAV2 serotypes. In addition, a structurally distinct AAV hybrid of the natural isolates rh.32 and rh.33 (L.H.V. and J.M.W., unpublished data) was included in this seroprevalence survey. These AAV variants were isolated from the spleen of a Rhesus macaque, and the amino acids encoded by their cap genes are ≈70% identical.
to those of AAV1, AAV2, AAV7, and AAV8 and 82% identical to those of their closest relative, AAV4 (figure 1A and figure 2, which is available only in the electronic version of the Journal). In comparison, the amino acids encoded by the cap gene of AAV1 are 83% homologous with those encoded by AAV8 cap [16].

MATERIALS AND METHODS

Cell culture. Human hepatoma cell line Huh7 was maintained in Dulbecco’s modification of Eagle’s medium (DMEM [Cell-gro]) supplemented with 10% fetal bovine serum (FBS [HyClone]). Cells were cultured at 37°C in an atmosphere of 5% CO2 in air.

Production of AAV vectors. AAV vectors contained the gene encoding β-galactosidase (LacZ), which was driven by a cytomegalovirus (CMV) promoter (AAV.CMV.LacZ). All AAV.CMV.LacZ vectors used in this study were made by the Vector Core of the University of Pennsylvania (Philadelphia, PA), as described elsewhere [17]. Recombinant AAV genomes equipped with AAV2 inverted terminal repeats (ITRs) were packaged by triple transfection of 293 cells with cis-plasmid, adenovirus helper plasmid, and a chimeric packaging construct in which the AAV2 rep gene was fused with cap genes of different AAV1, AAV2, AAV7, AAV8, and rh32.33 serotypes. All recombinant vectors were purified by the standard CsCl sedimentation method. Genome copy titers of AAV vectors were determined by TaqMan analysis (Applied Biosystems), using probes and primers targeting a bovine growth hormone polyadenylation signal, as described elsewhere [19].

Sources of serum samples. A total of 888 persons in 10 different countries across 4 continents participated in this study. Each person provided 1 serum sample. One hundred persons were from the Unites States (Department of Pathology and Laboratory Medicine, University of Pennsylvania Hospital [Philadelphia]), 100 were from Australia (National Centre for Immunization Research and Surveillance [Sidney]), 81 were from Greece (Clinical Microbiology, Evangelismos Hospital [Athens]), 100 were from Belgium (Rode Kruis Vlaanderen [Leuven]), 100 were from Italy (Telethon Institute of Genetics and Medicine [Napoli]), and 407 were from Africa, with 51 from Zambia (Lusaka), 51 from Kenya (Nairobi), 112 from South Africa (Cape Town), 60 from Rwanda (Kigali), and 113 from Uganda (60 from Kakira and 73 from Entebbe). All serum samples from persons in Africa were provided by Immunotherapeutics GlaxoSmithKline. No demographic information was provided for any of the persons from whom samples were obtained.

NAb assay. Serum samples were heat inactivated at 56°C for 35 min. Recombinant AAV.CMV.LacZ (10⁸ genomic copies/well) was diluted in serum-free DMEM and incubated with 2-fold serial dilutions (initial dilution, 1:20) of heat-inactivated serum samples on DMEM for 1 h at 37°C. Subsequently, the serum-vector mixture was added to 96-well plates seeded with 1 × 10⁵ Huh7 cells/well that had been infected 2 h earlier with wild-type HAdV5 (50 viral particles/cell). After 1 h, each well was supplemented with an equal volume of 20% FBS DMEM and incubated for 18–22 h at 37°C and 5% CO2. Then, cells were washed twice in PBS and lysed, and the lysate was developed with the mammalian β-galactosidase assay kit for bioluminescence, in accordance with the manufacturers’ protocol (Applied Biosystems), and measured in a microplate luminometer (Clarity [BioTek]). The NAb titer was reported as the highest serum dilution that inhibited AAV.CMV.LacZ transduction (β-gal expression) by ≥50%, compared with the mouse serum control (Sigma S3509).

Statistical and informatics analysis. Multiple logistic regression analysis was used to derive estimates of the prevalences of viral types in the continents from which samples were obtained. The analysis included an interaction between viral type and location to allow for regional differences within continents. The SEs were corrected to account for correlations between estimates for the same individual, and results were presented as prevalence estimates with 95% CIs. Excel (Microsoft) was used to measure the effect of sample bias on the linkage between the seroprevalences of different serotypes in a given population. The seroreactivity linkage ratio, created to describe the linkage in seroreactivity between 2 serotypes in a given population, is calculated by dividing the prevalence of serotype A in the subpopulation of persons who are positive for serotype B by the prevalence of serotype A in the subpopulation of persons who are negative for serotype B. Specifically, in each subpopulation, for a particular threshold of positivity (i.e., antiserotype NAb titers of 1:20 or 1:80), the seroreactivity linkage ratio between 2 serotypes A and B (denoted by RAB) was calculated as [PAB × NB]/[PA × PAB−], where N denotes the number of serum samples negative for serotype B, P denotes the number of samples positive for serotype B, PAB− denotes the number of samples negative for serotype B and positive for serotype A, and PAB+ denotes the number of samples positive for serotype B and positive for serotype A.

Comparison of amino acid sequences were done with ClustalX1.8, and phylogenies were inferred and visualized with MEGA4.0. All sequences are publicly available in Genbank (accession numbers AAV1-NP_049542, AAV2-YP_680426, AAV7-AAN03855, AAV8-AAN03857, and AAVrh.32.33-EU368926).

RESULTS

Serum samples from 888 persons spanning 4 continents and 10 countries were analyzed for the presence of NAb against AAV1, AAV2, AAV7, AAV8, and the structurally divergent vector rh32.33. NAb titers were determined for each sample; data are classified on the basis of cohort (i.e., geographic region) and the
prevalence of vector transduction inhibition at serum dilutions of 1:20 and 1:80. Figure 1 summarizes data for samples from persons in Australia, Europe, Africa, and the United States, figure 3 summarizes data for those in various African countries, and figure 4 summarizes data for those in different European countries.

Figure 1. Phylogenetic tree and prevalence of neutralizing antibodies (NAbs) against different adeno-associated virus (AAV) types in 100 serum samples from Australia, 281 from Europe, 407 from Africa, and 100 from the United States. A, Neighbor-joining phylogeny was inferred with Poisson correction for the protein sequences of the AAV VP1 Cap proteins. Scale, evolutionary distance of the number of substitutions per site. B and C, Samples were considered positive if serum dilutions of 1:20 (A) or 1:80 (B) inhibited vector transduction by ≥50%. **Less prevalent than anti-AAV2 NAb (P < .05); ***less prevalent than anti-AAV2 and anti-AAV1 NAbs (P < .05).

Seroprevalence of anti-AAV NAbs, by continent. The data were initially categorized on the basis of 4 large geographical areas: Australia (100 samples), Europe (281), Africa (407), and the United States (100). For all 4 cohorts, seroprevalences at serum dilutions of 1:20 (figure 1B) and 1:80 (figure 1C) were highest for anti-AAV2 NAb; the second highest seroprevalences...
were observed for anti-AAV1 NAb at each titer and for all cohorts except the >1:80 dilution in the United States. The differences between the seroprevalences of anti-AAV1 and anti-AAV2 NAbs were significant in Europe, Africa, and the United States at dilutions of >1:20 and in the United States at >1:80. For each dilution and all cohorts except the >1:80 dilution in the United States, for which the seroprevalence of anti-AAV7 NAb was second highest, the seroprevalences of anti-AAV7 and anti-AAV8 NAbs were lowest and were indistinguishable from one another ($P > .05$). In virtually all data sets, the seroprevalences of anti-AAV7 and anti-AAV8 NAbs were significantly different from the seroprevalence of anti-AAV2 NAb (Australia was an exception at >1:20) and, in most cases, from the seroprevalence of anti-AAV1 NAb. At >1:20, the highest seroprevalences of antibodies to each AAV were observed in Africa. The NAb titers in samples from each country cohort were also analyzed (figure 5). The trends noted in figure 1 are manifested in figure 5.

**Seroprevalence of anti-AAV NAbs, by country.** Analysis of the samples from Africa was further stratified into 6 regions: Entebbe, Uganda (73 samples), Kakira, Uganda (60), Rwanda (60), South Africa (112), Kenya (51), and Zambia (51). The seroprevalences of anti-AAVs NAbs are specified in figure 3A for serum dilutions of >1:20 and in figure 3B for serum dilutions of >1:80. Anti-AAV2 NAb had the highest seroprevalence, followed by anti-AAV1 NAb. However, the differences between anti-AAV2 and anti-AAV1 NAb seroprevalences were statisti-
cally significant in only 2 of 6 countries at a serum dilution of >1:20 and in only 1 of 6 countries at a dilution of >1:80. In all cases, the seroprevalences of anti-AAV7 and anti-AAV8 NAbs were statistically significantly lower than that of anti-AAV2 NAb, with more-dramatic differences at the higher dilution of >1:80 (the only exception was the seroprevalence of anti-AAV8 NAb at a dilution of >1:20 in Rwanda, where the difference was not statistically significant). Seroprevalences of anti-AAV7 and anti-AAV8 NAbs were statistically significantly lower than that for anti-AAV1 NAb in most countries at serum dilutions of >1:20 (in 4 of 6 countries for antibodies against both AAVs) and >1:80 (in 4 of 6 countries for anti-AAV7 NAb and 6 of 6 countries for anti-AAV8 NAb).

The situation in Europe (i.e., Belgium, Greece, and Italy) was similar at serum dilutions of >1:20, with the seroprevalence of anti-AAV2 NAb exceeding that of anti-AAV1 NAb (differences were statistically significant for samples from Italy and Greece). Data in Greece and Italy differed from findings in Africa at dilutions of >1:80: the seroprevalences of anti-AAV1 NAbs exceeded those of anti-AAV2 NAbs (figure 4). In Belgium, the seroprevalence of anti-AAV2 NAb was statistically significantly higher than that of anti-AAV1 NAb. The seroprevalences of anti-AAV7 and anti-AAV8 NAbs were lower than that of anti-AAV2 NAb in all countries at both serum dilutions. Differences between anti-AAV8 and anti-AAV2 NAb seroprevalences were statistically significant in 3 of 3 countries at a serum dilution of >1:20 and in 2 of 3 countries at a dilution >1:80, whereas differences between seroprevalences of anti-AAV7 and anti-AAV2 NAbs were significant in 3 of 3 countries at a dilution of >1:20 and in only 1 of 3 countries at a dilution of >1:80.

Relative degree of seroreactivity between AAV serotypes. We also wanted to know the percentages of individuals who had anti-AAV1, anti-AAV7, and/or anti-AAV8 NAb titters that were greater than the anti-AAV2 NAb titer. This information would be useful in assessing the relative value of using the novel serotypes rather than AAV2 as vectors. These data are summarized in table 1. In most areas, there were remarkably few individuals in whom the anti-AAV1, anti-AAV7, and/or anti-AAV8 NAb levels exceeded the anti-AAV2 NAb level.

Figure 4. Prevalence of neutralizing antibodies (NAbs) against different adeno-associated virus (AAV) types in 100 serum samples from Belgium, 81 from Greece, and 100 from Italy. Samples were considered positive if serum dilutions of >1:20 (A) or >1:80 (B) inhibited vector transduction by ≥50%. *Less prevalent than anti-AAV1 NAb (P < .05); **less prevalent than anti-AAV2 NAb (P < .05); ***less prevalent than anti-AAV2 and anti-AAV1 NAbs (P < .05).
The seroprevalence of anti-AAVrh32.33 NAbs. We recently created a novel AAV vector from a capsid formed as a hybrid between 2 similar capsids isolated from the spleen of a healthy-appearing Rhesus macaque. This capsid is called rh32.33 (figures 1 and 2). The same 888 serum samples that were used to screen for NAbs against AAV1, AAV2, AAV7, and AAV8 were evaluated for NAbs against AAVrh32.33. There was a remarkable absence of NAb against this capsid in all populations studied (figures 1, 3, and 4). Prevalence rates in any major region or the component countries were never

Figure 5. Global distribution and magnitude of the neutralizing antibody (NAb) response in serum samples from the United States, Australia, Europe, and Africa in which the NAb titer was ≥1:20. Each box encloses 50% of the data, with the upper and lower limits denoting the interquartile range; the horizontal line denoting the median value; the whiskers denoting minimum and maximum values in the data set that fell within an acceptable range; and the circles denoting outliers.
>2% in serum dilutions of >1:20 and were never detected in dilutions of >1:80.

**Seroreactivity linkage analyses.** An interesting question is whether there is a linkage in seroreactivity between 2 different AAVs within individuals. In other words if you are seropositive against one AAV, are you more likely to be seropositive against a second AAV? This analysis was performed as follows and is summarized in table 2. For example, we determined if a linkage existed between AAV7 and AAV8 in the United States by first dividing all those in the population into 2 groups: AAV7 seronegative and AAV7 seropositive (for purposes of this example the distribution was made based on titers >1:20). We then determined the frequency of seroreactivity at >1:20 to AAV8 in these 2 populations (AAV7 positive and AAV7 negative). Linkage is calculated by dividing frequency of AAV8-positive individuals in the AAV7 positive population by the frequency of AAV8-positive individuals in the AAV7-negative population (see Material and Methods). If there is no linkage for or against cross seroreactivity the ratio should be 1. A ratio of >1 suggests a positive linkage (i.e., seroreactivity to one virus is associated with a higher frequency of seroreactivity to another virus), whereas a ratio of <1 indicates a negative association (i.e., seroreactivity to one virus excludes reactivity to a second virus). In this example, for AAV7 versus AAV8, the ratio is 4.3. The reciprocal relationship (i.e., the increase in likelihood that AAV8-seropositive individuals are positive to AAV7 in comparison to the AAV8-seronegative individuals) yields a ratio of 12.8. A very different result is achieved when correlating seroreactivity between AAV2 and 7. In subjects from the United States their evaluation of AAV7-positive individuals who are positive to AAV2 yields a ratio of 1.1 with the reciprocal evaluation producing a ratio of 1.0. We concluded from this analysis that there is a high correlation between seroreactivity to AAV7 and AAV8 but not AAV7 and AAV2 in the United States. This analysis was performed for individuals from the 4 major geographical areas as presented in table 2 and further discussed below.

Europe and Australia produced high correlations in seroreactivity between different AAV serotypes. Very similar findings emerged from the analysis of samples from Africa with high correlations between all pairwise comparison except AAV2 in AAV1 positive (ratio, 2.2), AAV2 in AAV7 (ratio, 2.1) and AAV2 in AAV8 (ratio, 1.8). The data for the United States was indeed quite different, with most comparisons showing no correlations (AAV2 in AAV1 positive, AAV1 in AAV2 positive, AAV2 in AAV7 positive, AAV7 in AAV2 positive, AAV1 in AAV7 positive, and AAV7 in AAV1 positive). The comparisons of AAV2 and AAV8 yielded modest correlations (ratios, 3.0 and 1.8) with higher correlations between AAV7 and AAV8 as noted above (ratios, 4.3 and 12.8). It was interesting that there was a negative correlation between AAV1 and AAV8 (ratios, 0.3 for both pairwise comparisons).

**DISCUSSION**

Preexisting immunity to a specific AAV serotype due to a previous exposure from a natural infection can limit effective therapeutic gene transfer and efficacy of a genetic vaccine. For this reason, the study of the prevalence of NAbs to AAVs in human populations is critical for clinical vector development and the design of gene transfer and genetic vaccine regimens. This study is the largest published survey of seroprevalence of antibodies to different AAV types in worldwide human populations.

Our work demonstrated that the prevalence of anti-AAV2 NAb was significantly higher than that of other evaluated anti-
AAV NAb s in 10 countries across 4 continents. The prevalence ranged from 60% in Africa to 30% in the United States. A similar frequency for anti-AAV2 NAb was described previously in a US population [8, 20, 21]. Although the seroprevalence of antibodies to AAV1 was lower than that for anti-AAV2 NAb, it was still higher than those for anti-AAV7 and anti-AAV8 NAbs in most regions. This was clearly observed for samples with higher NAb titers. These data demonstrate a moderate yet significant advantage of AAV7 and AAV8 over AAV1 and AAV2 in overcoming preexisting B cell immunity in humans. Similar studies using
human pooled IgG (IVIG from 50,000 human plasma samples) have shown a 10-fold greater resistance of AAV8 to neutralization than AAV1 and AAV2 [22].

The low seroprevalence of antibodies to AAVrh32.33 is remarkable. AAV4, the relative of closest homology, was found equally resistant to human IVIG neutralization as AAV [23]. When AAVrh32.33 was tested against human IVIG, a 16-fold lower neutralizing activity than that for AAV8 was detected (table 3). Only samples with a wide breadth of neutralization to all tested serotypes and an AAV2 neutralizing activity of $\geq$1:2560 were reactive to this new AAV variant (data not shown). These data highlight the potential of this new hybrid AAV variant for human applications in broad populations. Although its properties are encouraging for development as gene therapy vectors, further studies are warranted to fully characterize its transduction properties and vector host interactions.

The remarkably higher frequency of anti-AAV2 NAb seropositivity observed in Africa than in other regions is surprising. This higher prevalence of NAB to AAV2 was accompanied by greater prevalences of NAbs to AAV1, AAV7, and AAV8 serotypes but not to AAVrh32.33. In the absence of clear demographic correlates to these data, it remains unclear whether living conditions, population density, hygienic conditions, MHC background, or other factors are involved in this phenomenon.

We observed a significant amount of linkage in seropositivity toward distinct serotypes in several evaluated populations and for several serotypes. One hypothesis to explain these results and the lack of a monospecific serological response is that the anti-serum raised in humans following infection with a single AAV serotype has a wide, cross-reactive repertoire. The different pattern of linkage in the United States, compared with the rest of the world, and the lack of serological cross-reactivity across serotypes of serum raised against AAVs in several animal models are not explained by this hypothesis. In another possible explanation, suggested by Halbert et al. [20], the cooccurrence of NAbs to multiple serotypes in the same individual may be the result of multiple subsequent or simultaneous infections with various AAV types in subpopulations at relatively higher risk for AAV infection. The apparent higher titers of anti-AAV2 NAb and other anti-AAV NAbs might then be explained by higher inherent immunogenicity [20] or multiple subsequent infections with the same serotype. Another hypothesis for these results is that humans generate a serological response of larger breadth that may be due to a rapid molecular evolution of multiple coexisting parental viruses in each individual: this could lead to the generation of antibodies directed against a wide spectrum of homologous antigens. This last hypothesis is supported by the wide distribution of heterogeneous AAV sequences retrieved from human tissue and the propensity of AAVs to evolve through various mechanisms of molecular evolution [14].

Recent unexpected results from the STEP HIV vaccine efficacy study led Moore et al. [23] to propose the existence of heterogeneity in the study populations with respect to the ability to mount an effective immune response, which may be relevant to our data. They pointed out an inverse correlation in the placebo group between preexisting NAb to adenovirus serotype 5 and acquisition of HIV infection and proposed that the level of antiadenovirus NAb is a surrogate measure of genetically determined stronger immune responses across a broad array of pathogens. The hypothesis of Moore et al. [23] suggests that individuals capable of generating more-robust immune responses would likely have more-robust NAb responses to AAV infections. Such infections, if prevalent (as suggested by our molecular epidemiology data), would lead to individuals with a broad profile of anti-AAV NAbs. The ability of the host to respond to infectious insults would be influenced greatly by genetic factors and therefore may exhibit regional variation, which is what we observed when comparing samples from the United States with samples from the rest of the world. It is also possible that linkage between AAV serotypes was not observed in the United States, because the overall frequency of serologic activity to AAV was lower.

Although the root of this wide breadth in NAb responses to AAV1, AAV2, AAV7, and AAV8 in humans is unclear, cross-reactivity toward AAVrh32.33 was only rarely observed, and when it was observed, the magnitude of the NAb response was much lower. AAVs that were isolated from mouse and rat were also more resistant than AAV2 to human antibody neutralization when tested by IVIG; of interest, vectors based on these viruses have limited tropism in human cell lines [18]. A novel caprine AAV that is capable of transducing human cell lines has been recently characterized by its resistance to neutralization by IVIG, but only to levels obtained by AAV4 and AAV8 [22].

Although in vitro detection of NAbs does not always mimic the mechanism by which NAbs may exert their effect in vivo, in this study we have demonstrated the minimal prevalence of NAb to a novel AAV vector, rh32.33, in several populations across the world. In addition, the lower frequency of NAbs to AAV7 and AAV8 serotypes may warrant their progression toward clinical application in light of the dominating global seroprevalence of antibodies to AAV2.

Table 3. Neutralizing antibody (NAb) titers of human pooled IgG in response to different adeno-associated virus (AAV) types.

<table>
<thead>
<tr>
<th>AAV type</th>
<th>NAb titer</th>
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<tbody>
<tr>
<td>AAV1</td>
<td>1:640</td>
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<tr>
<td>AAV2</td>
<td>1:2560</td>
</tr>
<tr>
<td>AAV7</td>
<td>1:640</td>
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<tr>
<td>AAV8</td>
<td>1:320</td>
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<tr>
<td>AAVrh32.33</td>
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