MOLECULAR ANALYSIS OF ADENOVIRUS
ISOLATES FROM PREVIOUSLY VACCINATED
YOUNG ADULTS

D. A. Blasiole
D. Metzgar
L. T. Daum
M. A. K. Ryan
J. Wu
C. Wills
C. T. Le
N. E. Freed
C. Hansen
G. C. Gray
K. L. Russell

Report No. 03-22

Approved for public release; distribution unlimited.
Molecular Analysis of Adenovirus Isolates from Vaccinated and Unvaccinated Young Adults

Daniel A. Blasie,* David Metzgar,1 Luke T. Daum,2 Margaret A. K. Ryan,1 Jianguo Wu,1 Christopher Wills,1 Charles T. Le,1 Nikki E. Freed,1 Christian J. Hansen,1 Gregory C. Gray,† and Kevin L. Rust2

Department of Defense Center for Deployment Health Research, Naval Health Research Center, San Diego, California 92186-5327; *Brooks City Base, San Antonio, Texas 78235; and Division of Biological Sciences, University of California, San Diego, La Jolla, California 92093

Received 25 September 2003;Returned for modification 10 October 2003;Accepted 25 November 2003

Infections of adenoviruses type 4 (Ad4) and Ad7 were discovered among previously vaccinated individuals through laboratory respiratory illness surveillance at military recruit camps. Genetic analysis was performed on those isolates and a sample of adenovirus isolates from unvaccinated patients. Antigenic regions of the adenoviruses hexon gene from 21 vaccinated and 31 unvaccinated patients were sequenced and compared to homologous regions of Ad4 and Ad7 vaccine strains and of other representative hexon sequences archived in GenBank. The phylogenetic distribution of sequences from vaccinated individuals closely resembled those from unvaccinated individuals. The most common Ad7 strain was the Ad7D hexon genotype, and the most common Ad4 strain was a genotype nearly identical to the recently discovered ZG-95-873 Ad4 variant. Near exclusive isolation of Ad4 since 1999 indicates that the Ad4 variant is currently responsible for the vast majority of adenovirus morbidity in military recruit camps. Different ratios of nonasymptomatous to asymptomatic nucleotide substitution rates in known antigenic regions compared to nonantigenic regions indicated positive selection for diversity in the antigenic regions and purifying selection in the nonantigenic regions.

Adenovirus was first discovered in 1953 (12, 22). Today, adenoviruses are categorized by their species (for- merly subgroups) (A to F), based primarily on differences in their hemagglutination properties, and by their serotype, based upon neutralization with type-specific animal antisera (17). There are currently 51 recognized serotypes of human adenovirus (95). The classical method for subtyping adenoviral isolates is whole-genome digestion via a stepwise, systematic restriction enzyme analysis (REA) process. Restriction enzyme classification methods use a numbering and lettering system appended to the serotype number to distinguish unique strains (16). The letters "A" through "K" represent restriction enzyme whole-genome electrophoretic banding patterns by the restriction en- zyme BamHI. An Arabic numeral is added when additional enzymes are used to further distinguish whole genomes. Intensively studied in the 1950s and 1960s, adenoviruses were found to infect up to 80% of military recruits and lead to hospitalization in up to 20% (9). Adenovirus type 4 (Ad4) and Ad7 were the primary serotypes responsible for this mor- tality and together constituted 60% of all hospitalized cases of acute respiratory disease (ARD) among military recruits (9). Live, enteric-coated oral vaccines, which induce immune re- sponses through selective infection of the gastrointestinal tract, were developed first for Ad4 and later for Ad7 (3, 28). These vaccines were shown to be safe and highly effective in the immunization of military trainees (28), and routine adminis- tration to recruits began in 1971 (10). The Ad4 and Ad7 vac- cines together lowered AV morbidity by 95 to 99% and total ARD morbidity by 50 to 60% during the period of vaccine use (10). However, the production of the vaccines was discontinued in 1996, and the remaining lots were retained until sup- ples were exhausted in early 1999 (10). Recently, the U.S. Department of Defense awarded a contract to Batt Labora- tories, Inc., for resumed production of the vaccines. Population-based military respiratory illness surveillance was initiated by the Naval Health Research Center in 1996 to document the epidemiology of adenoviruses during and after the period of vaccine loss (10). The surveillance program was originally established at four recruit training camps in the United States to define the burden of adenoviruses (10) but was later expanded to eight sites and included testing for other viral agents (24). This surveillance documented large increases in adenovirus morbidity and several fatal cases after the vac- cine was exhausted (2, 10, 25), suggesting that the initial vac- cine was efficacious for the majority of circulating pathogenic strains. However, several cases of Ad4 and Ad7 infection were discovered among previously vaccinated individuals, raising the possibility that newly emergent strains of adenoviruses had appeared.

Recent research on the evolution of circulating adenoviruses has engendered concern about the efficacy of the old vaccine against current strains. In order to determine the suitability of the original vaccine strains for a new vaccine, a study of strain variation among the circulating Ad4 and Ad7 serotypes was conducted in 1999 (5). The antigenic regions of the hexon gene from prototype, vaccine, community-acquired, and military wild-type strains collected from 1953 to 1997 were sequenced.

* Corresponding author. Mailing address: Naval Health Research Center, Department of Defense Center for Deployment Health Research, P.O. Box 85322, San Diego, CA 92120-5322. Phone: (619) 553-7626. Fax: (619) 553-7601. E-mail: nabell@nhrc.usmc.mil.
† Present address: College of Public Health, University of Iowa, Iowa City, IA 52242.
and compared. Whereas the hoxn antigen of Ad7 was generally conserved over time, an Ad7 variant (strain ZG 95-875) with nine amino acid changes in the hoxn antigen was found to have been circulating since 1995 (5). These changes were noted to confer decreased neutralization. The abilities of this strain to cause infection among vaccinated individuals, however, was not investigated. Another recent study analyzed Ad7 isolates from the United States from the years 1960 to 2000 by whole-genome REA (8). The study noted the appearance of two genome types previously undocumented in North America. Ad5d2 and Ad11, which indicated a shift in the predominant Ad7 genotype circulating in the United States. The hoxn protein of Ad5d2 contains only one unpaired amino acid substitution, but it has possible antigenic implications.

The primary objective of the present study was to determine whether the adenovirus infections among previously vaccinated individuals who had been between 1960 and 2000 resulted from prototype strains, recently discovered adenovirus variants, or a completely new variant. A 1,200-bp region of the adenovirus hoxn capsid protein gene contains seven discrete hypersensitive regions (HRVR to HVR7) that account for >99% of the sequence variability and code for the type-specific epitopes on the protein (4.5). This region of the hoxn gene from adenoviruses is isolated from vaccinated individuals and unvaccinated individuals was sequenced and compared to the vaccine strains. The AAd d variant strain, and the other Ad5 and Ad7 vaccine sequences in GenBank. The results of the present study will help guide vaccine development initiatives.

MATERIALS AND METHODS

Viral Health Research Center on satellite respiratory infections. The Naval Health Research Center has conducted population-based surveillance for upper respiratory infections in the United States since 1966. Present studies include Fort Jackson (South Carolina), Fort Leonard Wood (Missouri), Great Lakes Naval Training Center (Illinois), Marine Corps Recruit Depot (California), Fort Benning (Georgia), Lackland Air Force Base (Texas), Fort Irwin (California), and Camp May (New Jersey). Weekly rates of severe respiratory illness, defined as an oral temperature of >38 °C and either a cough or sore throat, are recorded. Records meeting the case definition are used to permit the collection of a throat swab specimen and to answer a medical questionnaire. Specimens are stored in sterile transport medium (Renn, Lanes, Kansas) and transported to the laboratory in Data to the Naval Health Research Center Respiratory Disease Laboratory, San Diego, Calif, for analysis. Classic viral culture and isolation are performed using M-MLV, II V (Synthetic Hybrid, Athens, Ohio) and A549 (American Type Culture Collection, Manassas, Va.) and PK15 (Dako, Denmark) cells. Cultures exhibiting viral growth are processed for identification of the infecting pathogen by using immunofluorescence. A medium selection of isolates positive for adenoviruses are further analyzed by immunoblotting with type-specific antisera to determine serotype as previously described (6).

Viral isolation, serology, adenovirus-positive samples, negative for other respiratory pathogens, were considered for inclusion in the study. In this manner, adenovirus was likely the causative agent of illness in the symptomatic cases at the time of collection. The vaccination status of the host (vaccinated or unvaccinated) and serotype (Ad5 or Ad7) as previously determined by immunoblotting, was then considered. Vaccinated individuals were defined as those with positive serology before January 15, 1990, after documented receipt of the Ad5 and Ad7 vaccine. The Ad7 sample set consisted of 10 isolates from vaccinated individuals and 30 from unvaccinated individuals and represent 4 of the 85 vaccine participants participating in the study. The Ad7 sample set consisted of 11 isolates from vaccinated individuals and 1 from an unvaccinated individual. A lack of evidence of Ad7 in recent years, with the exception of an outbreak at Great Lakes in the fall of 1997 (23), limited the availability of clinical isolates from unvaccinated individuals for this study. However, additional

<table>
<thead>
<tr>
<th>Primer</th>
<th>Genotype</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>R380</td>
<td>Hoxn</td>
<td>5'-ATTGGGAAGTGACCCACAG-3'</td>
</tr>
<tr>
<td>R222</td>
<td>Hoxn</td>
<td>5'-CACCCCTTTGCATGATGAC-3'</td>
</tr>
<tr>
<td>R440</td>
<td>Hoxn</td>
<td>5'-AAGGGGTGTTCTGGTCGGCC-3'</td>
</tr>
<tr>
<td>R737</td>
<td>Hoxn</td>
<td>5'-CGGTGGGAGTTCTACAGGC-3'</td>
</tr>
<tr>
<td>AYMHF</td>
<td>Hoxn</td>
<td>5'-GCAGTATCCGTCATTACGAC-3'</td>
</tr>
<tr>
<td>AYMR</td>
<td>Hoxn</td>
<td>5'-CTGTTTCAAGCTCACCCCTCG-3'</td>
</tr>
<tr>
<td>Ad5B1/S1</td>
<td>Fiber</td>
<td>5'-TATTGGGACACCTTCTCGCTCAAC-3'</td>
</tr>
<tr>
<td>Ad5B1/S1</td>
<td>Fiber</td>
<td>5'-GGCCGCTTCGACGAGACAGGCGCT-3'</td>
</tr>
</tbody>
</table>

sequences from GenBank supplemented the uncharacterized Ad7 sequences used in this analysis.

PCR amplification and sequencing. All selected samples were reboxed using primers in the original patient specimens. Viral DNA was extracted with the QIAamp DNA Blood Midi Kit (Qiagen, Valencia, Calif). The early conserved region and serotype-defining hypersensitive regions of the adenovirus hoxn, corresponding approximately to the 1,350 bp of the gene, was amplified by PCR with the primers UP1 and UP6, as previously described (5). The reaction mixture contained 1 × PCR Buffer II (Applied Biosystems, Foster City, Calif), 0.3 mM dNTPs, 200 μM concentrations of each dNTP (Applied Biosystems), and 1 μl of DNA template in a total volume of 20 μl. The cycling consisted of an initial denaturation at 94°C for 2 minutes, followed by 30 cycles of 94°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute, with a final extension of 72°C for 7 minutes. The amplicons were purified with QIAquick PCR purification kit (Qiagen).

Purified adenovirus genomic sequences were sequenced with the Perkin-Elmer/ABI Dye Terminator cycle sequencing Kit (Applied Biosystems). The sequencing consisted of 20 μl reactions of 10 μl, 5 μl, 5 μl, and 4 μl for 20 cycles. The products were purified using the Centrisep columns (Promega, Middleton, Wis). Baffled (55:45, v/v) was used for multiple sequence alignments. Sequences were analyzed using conserved conserved consensus.

Additional sequences for comparison. All available Ad5 and Ad7 sequences containing the 1,250-bp region of the hoxn gene in GenBank were added to our sequences for analysis. Four Ad7 sequences were added: the vaccine strain (AF166892); the BA-47 prototype (A019637); the newly described ZG 95-875 variant (AF436194), and a strain isolated in Korea (AF225322). Two Ad7 sequences were added: the vaccine strain (AF246807); three versions of the Genbank Ad7 prototype (AF006865, AF006871, AF006875); two variants of the 3H-385 Ad7 prototype (AF006866 and AF006865), the KY 98061-7 strain from a patient with a case of AIDS (AF006870), and the Ad7 prototype strain (AF031311). Eight isolates from Japan (AF031313) and 10 isolates from Thailand (AF031314, 15, 16, 17, 18, 19).

Analysis. Phylogenetic trees were calculated with the PHYLIP program version 3.5b28a (20) for all nucleotide and amino acid sequences by using neighbor-joining and maximum-parsimony methods. Several different tree construction strategies were used to test the robustness of the results and to search for possible relationships that exist among adenovirus serotypes. Sequences of adenovirus were compared to determine the evolutionary distance and the relationships among the different serotypes.
RESULTS

Surveillance serotype distribution. The proportional distribution of adenovirus serotypes isolated from military recruit camps from 1996 to 2002 differed markedly (Fig. 1). In 1996, during the last year of routine vaccine administration, Ad21 constituted the majority (58%, 14 of 24) of adenovirus morbidity, whereas Ad4 and Ad7 constituted only 4% each. Upon tapered use of the vaccine in 1997, however, Ad4 and Ad7 morbidity rose to 58% (899 of 1,568) and 26% (427 of 1,618), respectively, with other adenovirus serotypes constituting the remaining 6%. In 1998, Ad7 virtually disappeared, whereas Ad4 increased to 73% (191 of 260). The vaccine was completely depleted in 1999, with Ad4 responsible for 98% (247 of 248) of adenovirus morbidity. Ad4 remained at least at this proportion for the remainder of the analysis period with negligible representation from other serotypes.

Phylogeny and mutational characteristics. Phylogenetic analysis revealed a branching of the Ad7 sequences into two predominant groups. One group (Fig. 2, prototype group) contained the three human prototype sequences (only AAF095065 represented on the tree) and one military sample (S98-GL-96-V). The other group (Fig. 2, vaccine-strain group) contained the Ad7's vaccine strain, S-1998 Ad7 prototype, Ad7D prototype, one sequence from an unvaccinated military patient (S98-GL99-UV), ten sequences from vaccinated military patients, and three other sequences from Geckoskins.

The Ad7 vaccine-strain group was distinguished from the prototype group by 25 coding differences (Fig. 3a). The vaccine strain was identical to only one military strain (S98-GL-95-V), which was from a vaccinated individual. The other 10 military isolate sequences in this group differed by at least one amino acid. Seven of these contained an L439Q (HVR7) substitution, which was shared with the Ad7D2 strain and the prototype group (Fig. 3a). Of the 11 samples from vaccinated military patients, 7 carried L439Q.

Ad4 also divided into two main phylogenetic lineages. The first lineage (Fig. 2, vaccine-strain group) contained the Ad4 vaccine strain, the H1-67 prototype, and three military samples from 1998 and earlier (62-GL96-V, 62-FJ98-V, and 39-GL97-V). The second group (Fig. 2, variant group) contained the Z-G 95-873 variant, an isolate from Korea (AF542122), and the remaining 33 military isolates. All 3 military sample sequences in the vaccine-strain group were from vaccinated individuals compared to 4 of 33 in the variant group. The variant strain comprised 91.7% (33 of 36; 95% confidence interval, 78.2 to 97.1%) of all Ad4 sample sequences since 1996 and 100% (16 of 16; 95% confidence interval, 80.0 to 100%) since 1999.

The variant group was distinguished from the Ad4 vaccine-strain group by 10 amino acid substitutions (Fig. 3b). Of the 33 military samples in the variant group, 22 had identified amino acid sequences (Fig. 3b, sequences AX137244). Two exceptions (49-FLW97-V and 51-LACK96-NV) contained a nonconservative K216N substitution, as defined by differences in size and charge (4), which they shared only with the Z-G 95-873 variant and the vaccine strain (Fig. 3b).

Synonymous versus non synonymous substitutions. Pairwise comparison of conserved regions within each serotype yielded significantly greater mean synonymous substitution rates than mean non synonymous substitution rates (Table 2). In contrast, pairwise comparison of hypervariable regions within each serotype yielded greater mean non synonymous substitution rates than mean synonymous substitution rates. The differences between the means in the hypervariable regions was not significant; however, 103 of 231 independent pairwise comparisons among the Ad7 sequences had significantly greater non synonymous substitution rates, whereas only 10 had significantly greater synonymous substitution rates. Likewise, with the Ad4 sequences 319 of 780 comparisons had significantly greater non synonymous substitution rates, and 26 had significantly greater synonymous substitution rates. The large number of non significant pairwise comparisons was primarily the result of a high level of sequence redundancy in our data set.

Atrial strains. Four samples (three vaccinated and one unvaccinated), all isolated in 1998 and initially serotyped as Ad4, yielded mostly uncovetable nucleotide chromatographs,
FIG. 2. Ad4 and Ad7 hexon maximum-likelihood protein trees with canine Ad1 root. Isolates sequenced in the present study are labeled according to the following format: last two digits of accession number of identical sequence, location of isolation, sex of isolate, species name, and strain. FB, Fort Benning; P, Port Jackson; FLW, Fort Leonard Wood; LACK, Lackland; GL, Great Lakes; MCROD, Marine Corps Recruit Depot; V, vaccinated; UV, unvaccinated. Full accession numbers appear once in parentheses. Accession numbers in boldface correspond to those in Fig. 3. Multiple accession numbers among identical protein sequences are due to DNA sequences with silent mutations. Bootstrap values greater than 50 (of 100 total bootstraps) are shown. Rescaled consistency index = 0.97.
but short portions of readable fragments suggested the strains were Ads. Primers AdsHF and AdsHR were designed from the cleaved hexon segments, and primers AdsFIR-5F and AdsFIR- 5R (11) were designed from existing Ads sequences in GenBank. Both primer sets were used in sequencing, and the resulting sequences showed 100% identity (288 of 288 and 274 of 274, respectively) with Ads in GenBank (versions 2.25 and 2.26). Two further confirmatory tests included a type-specific PCR assay (19) and a second microradiation analysis, both of which verified the Ads diagnoses and showed no evidence of Ads coinfection.

DISCUSSION

Infection with mutant adenovirus strains constitutes one of the many possible reasons for vaccine failure. Improper storage or administration of vaccine, asymptomatic carriage of adenovirus that is not the cause of acute illness, or imperfect vaccine efficacy are all possible explanations for the isolation of adenoviruses among vaccinated individuals. With the development of a new adenovirus vaccine under way, however, it is vital to address the hypothesis of strain variation with these uniquely available isolates. Although it may be impossible to

| Table 2. Mean synonymous and non synonymous substitution rates in conserved and hypervariable regions of Ads7 and Ads4 |
| --- | --- | --- | --- |
| **Serotype** | **Conserved regions** | **Hypervariable regions** |
| | Synonymous | Non-synonymous | Synonymous | Non-synonymous |
| Ads7 | 39.9 ± 6.4 | 1.7 ± 0.6* | 26.6 ± 5.8 | 8.7 ± 2.3 |
| Ads4 | 17.6 ± 3.6 | 1.2 ± 0.4* | 10.2 ± 2.5 | 8.7 ± 2.3 |

* Rates were determined through pairwise sequence comparison and are expressed as synonymous substitutions per 1,000 synonymous sites and non-synonymous substitutions per 1,000 non-synonymous sites. **Significant difference between synonymous and non-synonymous substitution rates.**
determine whether one mutation or set of mutations was responsible for infection, consideration of these changes among circulating viral strains is an important step.

The major phylogenetic bifurcation within Ad7 hexon sequence was previously shown (5, 8, 14) and provided genetic evidence for a distinction originally seen only at a phenotypic level by cross-neutralization and REA (5). An elevatid reduction in neutralization of the Groman Ad7 prototype compared to the S-I0S Ad7a strain with Ad7a antiserum was also previously shown (5). This neutralizing difference may have contributed to a breakdown of the sole strain from a vaccinated patient in the prototype group (58, GL.90-V; Fig. 2), but the low number of prototype-like strains isolated in the present study indicated that they are not currently a major epidemiological threat in recruit camps.

A recent REA study on the 1997 Great Lakes outbreak showed >70% of the samples were of the Ad7/2D genome type (8). It was also found in the same study, as elsewhere (14), that an L445Q hexon substitution in HVR7 is consistently linked with Ad7/2 and Ad7f2, as distinct from Ad7a, Ad7b, Ad7c, Ad7g, and Ad7h. Of 10 samples in our study from the same outbreak but from vaccinated individuals, 7 also contained the L445Q substitution, indicating that these samples are most likely of the Ad7/2D genome type. The substitution is nonconservative and dramatically affects the hydrophobic and probably structural characteristics of the protein by transforming the nine-residue surrounding region in HVR7 from a secondarily structure conformation that exposes primarily hydrophobic amino acid residues into a conformation that exposes primarily hydrophilic residues (14). It has therefore been suggested that this substitution imparts antigenic implications (8), which may explain the high number of Ad7 of this type isolated from vaccinated individuals in our study. In contrast, however, evidence showing a rapid decrease in AV-associated illness after introduction of the vaccine in this outbreak (25) strongly suggests that the strain is at least partially susceptible to vaccine-induced immunity. Cross-neutralization data between Ad7a and a strain containing the L445Q substitution would provide more evidence of the antigenic implications.

Several other substitutions occurring among the Ad7s may have contributed to infection in vaccinated individuals. The nonconservative S425Y substitution in samples 56.9GL.79T and 57.9GL.97V (Fig. 3a) resided in HVR7 and may have directly altered the epitope. In addition, isolated nonconservative substitutions in nearly conserved regions may have indirectly affected the antigen. The known epitopes are known to be conformational, since only the protein in its native trimeric form and not linear hexon peptides or heat-denatured monomeric proteins neutralize in vitro (4). Therefore, a change in a structural region, such as the F080 substitution (Fig. 3a, 55.9GL.79T) in the conserved region at the base of the loop containing HVR7 (1, 4), for example, may drastically affect protein folding in the antigenic regions.

Given the predominance of Ad4 among cases of respiratory illness in recent years, as well as the predominance of the variant genotype within Ad4 (Fig. 1), it appears that this single strain was responsible for nearly all adenovirus-associated respiratory illness in military recruit camps since 1989. The continued increase in the dominance of this strain after vaccine cessation suggests that its proliferation was not primarily a result of vaccine use. It is difficult to say how well the prior vaccine strain will clinically protect against this variant, since the majority of the variant isolates were isolated after vaccine use was drastically reduced. However, it was recently shown in an in vitro cross-neutralization analysis that the Z-G 95-873 variant has a furthid reduction in neutralization in comparison with the Ad4 Rv-67 prototype strain (5), a strain similar in genotype to the vaccine strain. The majority (32 of 35) of genotypes in the variant group differed from the Z-G 95-873 variant, as well as the vaccine strain, by the N226K amino acid substitution (Fig. 3b, sequence AT33274). If this substitution changes the epitope at all, it would render it further unrecognized by vaccine-induced antibodies; however, it is unknown whether the substitution would have such an effect, given its nonconservative character in a conserved region. Nevertheless, due to the current prevalence of the Ad4 variant with the N226K mutation, any new vaccine should be optimized for effectiveness against this strain.

As with Ad7, several isolated substitutions in the Ad4 set have the potential to alter the epitope. Three nonconservative amino acid changes (Fig. 3b, P320T in sample 39.9GL.79T, N246K, and S420F) resided in antigenic regions. Nonidentical nucleotide mutations code for the N226K substitution, which suggests convergent evolution at this site, perhaps driven by selection. However, given that only one sample from a vaccinated individual was found with each mutation, our data do not conclusively show their clinical effects.

Nucleotide sites are classified as either non synonymous or synonymous depending on whether or not, respectively, a mutation at the site will lead to an amino acid substitution. The first and second nucleotide sites in a codon are largely non synonymous, and the third site, due to redundancy in the genetic code, is largely synonymous. The degree to which a site is classified as either synonymous or non synonymous is determined by accounting for the coding impacted by all possible mutations. Within a gene region, the number of non synonymous substitutions per synonymous site relative to synonymous substitutions per synonymous site indicates the type of evolutionary selective pressure on the region. Equal rates of non synonymous and synonymous mutations indicate neutral drift, an excess of synonymous substitution rates indicates positive selective pressure, and an excess of non synonymous substitution rates indicates selection for diversity (diversifying selection). Evidence of diversifying selection is often found in protein regions that benefit from variation such as surface antigens of pathogens (7) and in immunoglobulin variable regions (27). In our study, the non synonymous substitution rates in the antigenic (hypervariable) regions of the sequences suggest that these regions are affected by diversifying selection. In contrast, the nonantigenic conserved regions show strong purifying selection, as is expected from regions with a structural function. Given the propensity for variation in the hypervariable regions, one would expect that the selective pressure of vaccine effector cells might eventually allow the virus to evolve resistance to vaccine-induced immune responses. Antigenic cross-neutralization analysis between new vaccine strains and currently circulating strains should be closely monitored.

A recent study of the adenovirus hexon (23) proposed a reassignment of the hypervariable regions based on "creches"
of density in an alignment of 40 partial hexon amino acid sequences from both human and simian adenoviruses.

The hypervariable regions as presented in the alignment in the study generally overlap with the assignments as previously defined for Ad7 and Ad27 (5). An analysis of mutation rates in the reassembled hypervariable regions of the sequences used in our study shows higher synonymous than non-synonymous substitution rates in the hypervariable regions (data not shown), which is the opposite of what was found with the previous assignments. Additional support for the previous region assignments is provided by a visual inspection of the alignment used to define the new assignments. This reveals that definite motifs in a subset of sequences, including that of Ad27, are shifted out of phase with those of Ad4, Ad7, and several others. Such a shift would affect identity scores assigned to each region and hence the placement of hypervariable regions in the alignment. Our data therefore support the hypervariable positions as previously defined for Ad4 and Ad7.

Initiation of the Ad5 vaccine in the 1960s was associated with Ad5 and Ad21 emerging as the chief etiological agents of ARD in military recruit camps. At this time, the constellation of the first outbreak of Ad21 in the Western Hemisphere (21). The isolation of three of the four Ad5 strains from vaccinated individuals indicates that this serotype may have similarly filled an evolutionary niche left open during the period of Ad5 and Ad7 vaccine use, but to a much smaller extent. Recent evidence shows that Ad5 manifests a lower binding affinity and replicates more slowly in A549 cells relative to other adenoviruses in subgroups A, B (includes Ad57), D, E, and E (includes Ad45) (19). Ad549-mediated growth typically used in the serological diagnosis of Ad5 may fail to produce an Ad5 site sufficient for detection by monoclonal antibodies. The epidemiological impact of Ad5 on military recruit populations during the period of vaccine use may therefore have been underestimated. Molecular surveillance should be used to monitor for the possible emergence of Ad21, Ad5, or other serotypes upon reintroduction of the Ad5 and Ad7 vaccines.

Although the current study cannot definitively explain the reason for past vaccine failures, it shows the distribution of strains with possible partial resistance to the previous vaccines. Given the predominance of the Ad5 variant in recent years, an effective vaccine against it may significantly reduce adenovirus-associated respiratory infection in the military. Although Ad5 is not circulating at present, protection against the Ad542 strain would prevent any opportunistic proliferation upon controlling Ad5. Other serotypes have not shown Ad5's capacity to fill the Ad5 niche, but, with diversifying selection acting on the hexon epitopes, continued surveillance for new emergent strains should be conducted.

Acknowledgments

This report represents 03-27 and was supported by the U.S. Department of Defense and the National Institutes of Health. The views expressed here are those of the authors and do not reflect the official policy or position of the Department of the Navy, the Department of Defense, or the U.S. Government. The work is approved for public release (distribution unlimited). The research has been conducted in compliance with all applicable federal regulations governing the protection of human subjects in research under protocol 52627.

We thank Anthony W. Hawksworth of the Department of Defense Centers for Deployment Health Research for organization and analysis of fibrous tissue surveillance data. We also thank Leila K. Crawford-Myers and Donald P. Schimmer of the Vital and Rekall Ulcer Disease Laboratory, Division of Communicable Disease Control, California State Department of Health Services, for adenovirus analyses and expert consultation.

REFERENCES


REPORT DOCUMENTATION PAGE

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB Control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. Report Date (DD MM YY) 28 August 2003

2. Report Type New

3. DATES COVERED (from - to) 1996-2000

4. TITLE AND SUBTITLE Molecular Analysis of Adenovirus Isolates From Previously Vaccinated Young Adults.


6. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Chief, Bureau of Medicine and Surgery Code M2 2300 E St NW Washington DC 20372-5300

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Naval Health Research Center P.O. Box 85122 San Diego, CA 92186-5122

8. SPONSORING/MONITORING AGENCY REPORT NUMBER Report No. 03-22

9. SPONSOR/MONITOR'S ACRONYM(S) UNCL

10. SPONSOR/MONITOR'S REPORT NUMBER(S) UNCL

11. SUPPLEMENTARY NOTES Published in: Journal of Clinical Microbiology, 2004, 42(4), 1686-93

12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited.

13. SECURITY CLASSIFICATION OF: a. REPORT UNCL b.ABSTRACT UNCL c. THIS PAGE UNCL

14. ABSTRACT (maximum 200 words) Infections of adenovirus type 4 (AV4) and type 7 (AV7) were discovered among previously vaccinated individuals through febrile respiratory illness surveillance at military recruit camps. Genetic analysis was performed on these isolates and a sample of adenovirus isolates from unvaccinated patients. Antigenic regions of the adenovirus hexon gene from 21 vaccinated and 31 unvaccinated patients were sequenced and compared with homologous regions of AV4 and AV7 vaccine strains and of other representative hexon sequences archived in GenBank. The phylogenetic distribution of sequences from vaccinated individuals closely resembled those from unvaccinated individuals. The most common AV7 strain was the AV7d2 hexon genotype, and the most common AV4 strain was a genotype nearly identical to the recently discovered Z-G 95-873 AV4 variant. Nearly exclusive isolation of AV4 since 1999 indicates that the AV4 variant is currently responsible for the vast majority of adenovirus morbidity in military recruit camps. Different ratios of nonsynonymous to synonymous nucleotide substitutions in known antigenic regions compared with nonantigenic regions indicated positive selection for diversity in the antigenic regions and purifying selection in the nonantigenic regions.

15. SUBJECT TERMS adenovirus-4, adenovirus-7, hexon gene, respiratory illness, vaccines, sequencing, military recruits

16. SECURITY CLASSIFICATION OF: a. REPORT UNCL b.ABSTRACT UNCL c. THIS PAGE UNCL

17. LIMITATION OF ABSTRACT UNCL

18. NUMBER OF PAGES 8

19. NAME OF RESPONSIBLE PERSON Commanding Officer

19b. TELEPHONE NUMBER (INCLUDING AREA CODE) COMM/DSN: (619) 553-8429

Standard Form 298 (Rev. 8-98) Prescribed by ANSI Std. Z39-18