Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 6763–6767, September 1989
Medical Sciences

Immunogenicity and efficacy testing in chimpanzees of an oral hepatitis B vaccine based on live recombinant adenovirus

(adenovirus animal model/adenovirus-vectored vaccines)

MICHAEL D. LUBECK*, ALAN R. DAVIS*, MURTY CHENGALVALA*, ROBERT J. NATUK*, JOHN E. MORIN*, KATHERINE MOLNAR-KIMBER*, BRUCE B. MASON*, BHEEM M. BHAT*, SATOSHI MIZUTANI*, PAUL P. HUNG*, AND ROBERT H. PURCELL†

*Wyeth–Ayerst Research, Biotechnology and Microbiology Division, P.O. Pox 8299, Philadelphia, PA 19101; and †Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

Contributed by Robert H. Purcell, May 30, 1989

ABSTRACT **As a major cause of acute and chronic liver disease as well as hepatocellular carcinoma, hepatitis B virus (HBV) continues to pose significant health problems worldwide. Recombinant hepatitis B vaccines based on adenovirus vectors have been developed to address global needs for effective control of hepatitits B infection. Although considerable progress has been made in the construction of recombinant adenoviruses that express large amounts of HBV gene products, preclinical immunogenicity and efficacy testing of candidate vaccines has remained difficult due to the lack of a suitable animal model. We demonstrate here that chimpanzees are susceptible to enteric infection by human adenoviruses type 7 (Ad7) and type 4 (Ad4) following oral administration of live virus. Moreover, after sequential oral immunization with Ad7- and Ad4-vectored vaccines containing the hepatitis B surface antigen (HBsAg) gene, significant antibody responses to HBsAg (anti-HBs) were induced in two chimpanzees. After challenge with heterologous HBV, one chimpanzee was protected from acute hepatitis and the other chimpanzee experienced modified HBV-induced disease. These data demonstrate the feasibility of using orally administered recombinant adenoviruses as a general approach to vaccination.**

Hepatitis B is a serious health problem worldwide that afflicts more than 300 million individuals, with 0.5–1 million new cases appearing annually in China alone (1, 2). To address the requirements for an inexpensive, effective, and easily administered hepatitis B vaccine, live recombinant hepatitis B vaccines using adenovirus vectors have been developed. The adenovirus vector approach is based, in part, on the successful application during the past 2 decades of live adenovirus vaccines for prevention of acute respiratory disease in military trainees (3, 4). Selective infection of the intestine with orally administered adenovirus types 4 and 7 (Ad4 and Ad7) vaccines has been highly effective in suppression of acute respiratory disease in military recruits, which is caused primarily by these two adenovirus serotypes.

Experimental adenovirus-vectored hepatitis B vaccines have been described (5–8), but preclinical testing of these vaccines in animals has been difficult due to the highly restricted host range of human adenoviruses. Although models for disease induced by adenoviruses have been sought since their discovery over 3 decades ago, only recently have the cotton rat (9) and the hamster (7, 10) been identified as models for adenovirus-induced respiratory infections. Although these rodent species support lung replication of various adenovirus serotypes, these animals are not susceptible to infections by Ad4 or Ad7 (ref. 9; unpublished data), which, because of their wide prior usage, are good candidates as vectors. Other less well-characterized small animal models for human adenoviruses have been occasionally reported (11–13) but are likewise nonpermissive for Ad4 and Ad7 infections (unpublished data). An early study of animal species including nonhuman primates indicated that human adenoviruses do not induce acute respiratory disease in monkeys or chimpanzees following intranasal inoculations (14). Serological data, however, indicated that such experimental infections occasionally induced anti-adenovirus antibodies in chimpanzees but not in monkeys.

In the present study, chimpanzees were evaluated for enteric infection by Ad4 and Ad7. We also investigated whether chimpanzees sequentially immunized with Ad7- and Ad4-vectored hepatitis B vaccines develop significant anti-hepatitis B surface antigen (HBsAg) responses and whether such responses are protective in chimpanzees after hepatitis B virus (HBV) challenge.

MATERIALS AND METHODS

Cells and Viruses. Cell line A549 derived from human lung carcinoma was used for calcium phosphate transfection as described (6). The vaccine strains of Ad4 (CL68578) and Ad7 (55142) and the recombinant adenoviruses described below were grown and titrated on A549 cells as well as on the human diploid cell strain WI-38 (15).

Construction of Recombinant Viruses. The recombinant adenoviruses described here were constructed by the same methods used to construct the recombinant adenovirus type 5 viruses previously described (6). The HBsAg gene (extending from nucleotide 130 to 966; *adw2* subtype) was inserted into a cloned fragment of the Ad7 genome that extended from Ad7 map unit (m.u.) 68 to m.u. 100, as shown in Fig. 1. In the resulting plasmid, pAd7H, that portion of the Ad7 E3 region between m.u. 80 and m.u. 84 was deleted, and the HBsAg gene was inserted at the site of this deletion so that the translation initiation codon of HBsAg was 829 nucleotides downstream of the TATA box of the E3 region promoter. This deletion completely removes the sequence encoding the Ad7 19-kDa glycoprotein (gp19K), the principal protein product of the E3 region (16). When this recombinant plasmid was transfected into A549 cells together with Ad7 DNA, the recombinant virus Wy-Ad7HZ6-1 was generated by homologous recombination (Fig. 1). HBsAg was secreted into the medium by cells infected with Wy-Ad7HZ6-1 and was found to consist of spherical particles (predominantly 20–25 nm) indistinguishable from particles found in the serum of chronic

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HBV, hepatitis B virus; HBsAg, HBV major surface antigen; Ad4 and Ad7, adenoviruses type 4 and 7, respectively; ALT, alanine aminotransaminase; m.u., map unit(s); anti-HBc, antibodies to HBV core antigen.

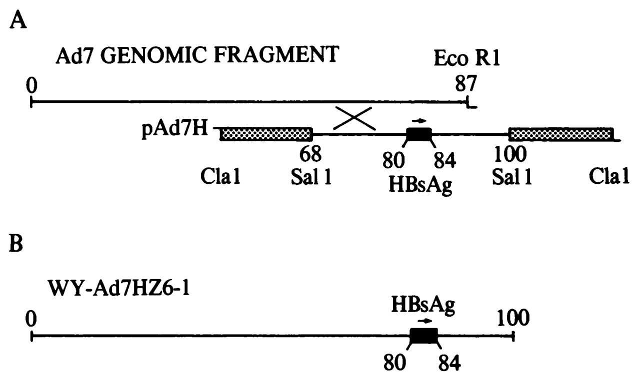

FIG. 1. (*A*) The recombinant adenovirus Wy-Ad7HZ6-1 was constructed by transfecting into cultured A549 cells the large *Eco*RI fragment of Ad7 genomic DNA together with a partially overlapping fragment of cloned Ad7 DNA from a plasmid (pAd7H; stippled boxes represent flanking plasmid DNA). (*B*) Wy-Ad7HZ6-1 was isolated after transfection and identified by screening for HBsAg production using a RIA.

HBV carriers. The secreted particles contained p24 and gp27 polypeptides (data not shown). The total yields of HBsAg produced in tissue culture were typically 0.8–1.0 μg per 10^6 cells infected.

The Ad4 recombinant used in this study (Wy-Ad4HHxHS) was engineered from the vaccine strain of Ad4 by insertion of the HBsAg gene (nucleotides 130–966) 786 base pairs downstream of the E3 region TATA box. None of the E3 region was deleted.

Capsule Production. A clarified Wy-Ad7HZ6-1-infected cell lysate was concentrated by polyethylene glycol precipitation (PEG 8000; final concentration, 12.5%) and infectious virus was separated from HBsAg by centrifugation (SW 50.1 rotor; 35,000 rpm; 90 min) on a discontinuous CsCl gradient [CsCl at 1.3 g/ml and 1.4 g/ml in 0.05 M Tris·HCl (pH 7.5)]. The virus preparation was dialyzed against 0.01 M Tris buffer (pH 7.5) containing 0.29 M NaCl and was subsequently shown by Ausria (Abbott) to contain <2.0 ng of HBsAg per ml. The virus preparation was diluted ≈1:10 with Dulbecco's modified essential medium (GIBCO) supplemented with 0.22 M sucrose, 0.044 M potassium phosphate (monobasic), 0.007 M potassium phosphate (dibasic), and 0.005 M potassium glutamate. The preparation was then lyophilized and stored at −70°C. Hard gelatin capsules were filled with a predetermined amount of lyophilized material, after which the capsules were enteric-coated (six coats) with cellulose acetate phthalate [10% (wt/vol) in acetone/ethanol, 50:50]. Enteric-coated capsules containing Ad7 and Wy-Ad4HHxHS were prepared as described above except that viruses were obtained directly from infected cell lysates. Capsules prepared from Wy-Ad4HHxHS-infected cell lysates contained <400 ng of HBsAg. After inoculation of chimpanzees, unused capsules were titrated following solubilization of lyophilized material in phosphate-buffered saline.

Chimpanzee Inoculations. Chimpanzee immunogenicity and challenge studies were conducted at Sema (Rockville, MD). Three males (nos. 1373, 1374, and 1376) and one female (no. 1375) 1–2 years old were selected for use in the study based on an adenovirus antibody screen (see *Results*). The chimpanzees were housed in individual isolators throughout the study and were maintained as described (18). Prior to vaccination, a preliminary study was conducted to evaluate dissolution in the chimpanzee gut of enteric-coated capsules containing barium sulfate (capsules were prepared as indicated above). Serial roentgenograms indicated that the capsules disintegrated in the small or large bowel within 24 hr of capsule administration. For primary and booster immunizations, chimpanzees were given one or two enteric-coated capsules through a gastric tube after anesthetization with ketamine. Two hours after booster vaccinations, the chimpanzees were given 60 ml of 0.10 M phosphate buffer (pH 8.0) to promote dissolution of the enteric-coated capsules in the chimpanzee gut. Seven weeks after booster inoculations, the three vaccinated chimpanzees and a control chimpanzee (no. 1376) received an intravenous challenge with $10^{3.5}$ 50% chimpanzee infectious units of HBV (subtype *ayw*, strain MS-2) (18).

Detection of Shed Virus. Stool samples were made 10% (vol/vol) in Dulbecco's modified essential medium containing 2% penicillin/streptomycin and clarified by centrifugation prior to inoculation on A549 cell monolayers. Cultures not displaying adenovirus cytopathic effect by 10 days postinoculation were given a single blind passage and observed for an additional 10-day period. The presence of adenovirus antigen in cell culture fluids was confirmed by a monoclonal antibody-based enzyme linked immunosorbent assay (19). The rabbit anti-Ad2 hexon antiserum and anti-Ad3 hexon monoclonal antibody (20/11 MAF) used in this assay were gifts of John C. Hierholzer (Centers for Disease Control, Atlanta, GA). Isolates were identified as adeno–hepatitis recombinants by evaluation of infected-cell culture fluids for HBsAg by RIA (Ausria II-125). After booster inoculations, the Ad4 serotype of adenovirus isolates was confirmed by neutralization analysis using type-specific polyclonal rabbit antisera.

Serology. Chimpanzees were bled at weekly intervals and sera were tested for anti-HBs antibodies by RIA (Ausab, Abbott). Anti-HBs responses are expressed in milliinternational units (mIU), based on a conversion factor of 3.5 RIA units per 1 mIU). Sera were further tested for HBsAg by RIA (Ausria II-125), for anti-HBc (HBV core antigen) antibodies by a competition RIA (Corab, Abbott), alanine aminotransaminase (ALT) (20), and anti-adenovirus antibodies by microneutralization analysis on A549 cell monolayers using a challenge dose of 30–100 50% tissue culture infectivity doses (8).

RESULTS

Seroprevalence Study in Chimpanzees. Sera from 31 chimpanzees were screened for type-specific neutralizing antibodies to human Ad4 and Ad7. Although these chimpanzees were generally seronegative for anti-Ad7 antibodies (reciprocal titers, <4), 77% of the animals had significant anti-Ad4 antibody titers (reciprocal titers, >4) (Table 1). Chimpanzees 1373, 1374, 1375, and 1376 were selected for use in this study on the basis of low neutralizing antibody profiles for both Ad4 and Ad7.

Clinical Observations Immediately Following Vaccination. Chimpanzees received primary oral immunizations with Ad7 or Wy-Ad7HZ6-1 followed 11 weeks later by oral booster vaccinations with Wy-Ad4HHxHS as indicated in Table 2. The chimpanzees remained healthy throughout the 2-week

Table 1. Incidence of anti-Ad4 and anti-Ad7 neutralizing antibodies in chimpanzee sera

	Antibody titer (reciprocal)	
% sera	Ad4	Ad7
23	<4	<4
23	4	<4
16	8	<4
13	16	<4
13	32	<4
10	64	<4
3	>128	>128

period immediately following the primary inoculations. No adverse respiratory, enteric, or other clinical reactions were observed in any of the chimpanzees during this period. During the third week postinoculation, chimpanzees 1373 and 1375 contracted intercurrent upper respiratory tract infections, which were shown to be unrelated to vaccine administration. During a 3-week period starting at 4 weeks postinoculation, chimpanzee 1373 experienced slightly elevated serum levels of liver enzymes (1.5–3 times normal levels for ALT and other transaminases). A biopsy of the liver of this animal at 5 weeks postinoculation presented no evidence of pathology upon histological evaluation and failed to yield infectious virus upon cultivation of biopsy material in cell culture. No other adverse clinical effects were observed in these chimpanzees during the balance of the 11-week primary vaccination period. The chimpanzees also remained healthy without observable enteric or respiratory disease throughout the 7-week period following booster inoculations.

Virus Recovery. Shedding of infectious virus in stools was monitored by recovery of virus in cell culture. Fig. 2 indicates that after primary vaccinations all three chimpanzees shed virus for 6–7 weeks. Viruses shed in stools of chimpanzees 1373 and 1374 were confirmed as Wy-Ad7HZ6-1 by detection of HBsAg produced in infected cell culture lysates. Positive cultures for chimpanzee 1375 were negative for HBsAg production. The period of time that virus was shed at high titer ($\geq 10^3$ infectious virus per ml of stool) for these three animals after primary immunizations was proportional to the dose of administered virus.

After booster inoculation of chimpanzees with Wy-Ad4HHxHS, infectious virus was excreted in stools for 17–34 days. Shed adenovirus was confirmed as Wy-Ad4HHxHS by neutralization analysis with an Ad4-specific antiserum and by detection of HBsAg in infected cell culture lysates (data not shown).

Serological Responses. After primary oral inoculations, each of the three chimpanzees developed significant anti-Ad7 neutralizing antibody responses (reciprocal titers, 16–64) by 3 weeks postinoculation (Fig. 2). Anti-Ad7 titers persisted at high levels throughout the remainder of the study (11 months) for all three chimpanzees. Each of the three chimpanzees experienced significant anti-Ad4 neutralizing antibody responses (reciprocal titers, 64) within 3 weeks of booster immunizations with Wy-Ad4HHxHS (Fig. 2). Anti-Ad4 neutralizing antibody titers peaked at ≈2 months and remained elevated throughout the experiment.

After primary immunization with Wy-Ad7HZ6-1, chimpanzee 1374 developed a transient seroresponse to HBsAg, which peaked at 9 mIU during the fifth week postinoculation. The anti-HBs response then declined during the following 2-week period. Chimpanzee 1373, which was also vaccinated with Wy-Ad7HZ6-1, did not mount a detectable anti-HBs response. Following booster immunizations of chimpanzees 1373 and 1374 with Wy-Ad4HHxHS, secondary anti-HBs responses were elicited. The anti-HBs booster responses developed more rapidly, persisted for longer periods, and peaked at higher levels (Fig. 3) than the primary anti-HBs responses. Chimpanzee 1375, whose vaccination with Wy-Ad4HHxHS represented a primary vaccination with an adeno–hepatitis recombinant, did not develop a detectable anti-HBs response.

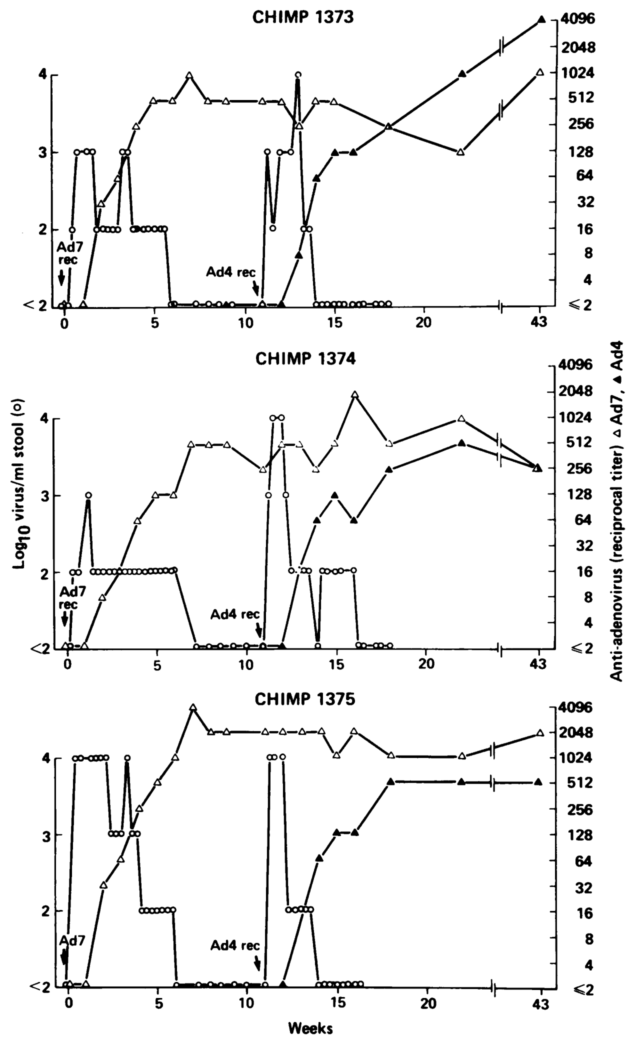

FIG. 2. Shedding of adenoviruses in stools and anti-adenovirus neutralizing antibody responses in chimpanzees. Chimpanzees received primary inoculations on day 0 and booster inoculations during week 11 as indicated in Table 2. Virus was detected in stool by culturing in A549 cells. Neutralizing antibody responses are expressed as reciprocal titers.

HBV Challenge. Eight weeks after booster immunizations, chimpanzees 1373, 1374, and 1375, as well as a control chimpanzee (no. 1376), were challenged with $10^{3.5}$ chimpanzee infectious doses of heterologous HBV (*ayw* strain). Ten weeks after challenge, the control chimpanzee (no. 1376) experienced an acute HBV infection. HBsAg was detected in the blood of this animal for an 11-week period and elevated serum levels of liver enzymes followed a course typical for an acute HBV infection (Fig. 3). Anti-HBc and anti-HBs re-

Table 2. Schedule for immunization of chimpanzees with recombinant adeno–hepatitis virus and subsequent challenge with HBV

	Primary immunization		Booster immunization			HBV challenge	
Animal	Virus	Dose, pfu	Virus	Dose, pfu	Weeks post-prim. immun.	Dose*	Weeks post-prim. immun.
1373	Wy-Ad7HZ6-1	3×10^7	Wy-Ad4HHxHS	8×10^9	11	$10^{3.5}$	18
1374	Wy-Ad7HZ6-1	1.5×10^7	Wy-Ad4HHxHS	8×10^9	11	$10^{3.5}$	18
1375	Ad7	1×10^9	Wy-Ad4HHxHS	8×10^9	11	$10^{3.5}$	18
1376	—	—	—	—	—	$10^{3.5}$	—

pfu, Plaque-forming units; post-prim. immun., post-primary immunization.
*50% chimpanzee infectious units.

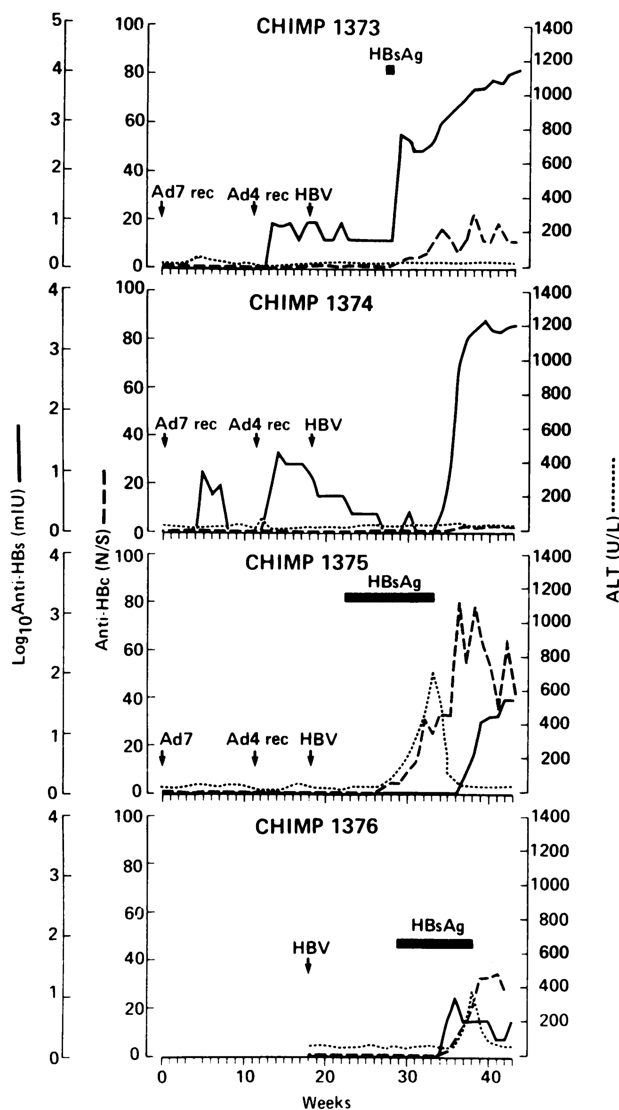

FIG. 3. Response of chimpanzees to vaccination with Ad7 and recombinant adenoviruses followed by challenge with HBV. Chimpanzees were vaccinated and then challenged with HBV as indicated in Table 2. Anti-HBs responses are expressed as mIU/ml and anti-HBc responses are expressed as the ratio of negative control cpm to sample cpm (N/S). Positive HBsAg values (positive/negative, >2.1) are shown as solid bars. Peak values were 46.4, 166, and 161 for chimpanzees 1373, 1375, and 1376, respectively. Serum ALT activity is expressed in IU/liter.

sponses developed several weeks after initial detection of HBsAg in the blood.

Chimpanzee 1374, which experienced the highest anti-HBs response after dual vaccination and which had a titer of 9 mIU at the time of HBV challenge, did not develop clinical hepatitis. HBsAg was not detected in the blood of this chimpanzee and liver transaminase levels were normal throughout the 6-month observation period following challenge. However, 17 weeks postchallenge, a strong anti-HBs response developed as well as a slight but significant anti-HBc response. This increase, which ranged from 2.4 to 3.5 negative/positive units from weeks 36–43, is not apparent in Fig. 3. Values ≥2.1 are considered positive. The dramatic anti-HBs response consisted primarily of antibodies of the IgG isotype (data not shown). The combination of the newly developed anti-HBc response and the anamnestic anti-HBs seroresponse indicated that chimpanzee 1374 experienced a subclinical HBV infection.

Chimpanzee 1373, which also possessed an anti-HBs serum titer of 9 mIU at the time of HBV challenge, also developed a subclinical HBV infection. Low levels of HBsAg were detected in the serum of this animal for a brief 2-week period starting at 9 weeks postchallenge. However, no elevation in liver enzyme levels was observed in this animal. Immediately after the transient antigenemia, a large anti-HBs response developed. This response had not yet peaked at the time the experiment was terminated and was also of the IgG isotype (data not shown). An anti-HBc antibody response developed 11 weeks postchallenge.

Chimpanzee 1375, which did not develop a detectable anti-HBs response following vaccination with Wy-Ad4-HHxHS, was not protected from clinical hepatitis following HBV challenge.

DISCUSSION

Our results indicate that the chimpanzee is susceptible to enteric infection by wild-type Ad7 and recombinant Ad7 and Ad4. We also demonstrate that sequential oral immunization of the chimpanzee with Ad7- and Ad4-vectored adeno–hepatitis viruses containing the HBsAg gene induced a low but significant anti-HBs humoral immune response. Primary vaccination of two chimpanzees with Wy-Ad7HZ6-1 resulted in a detectable but transient anti-HBs response in one animal and a priming anti-HBs response in the other animal. These low primary responses are consistent with the relatively poor immunogenicity of HBsAg in primates (21). Following booster immunizations of these two chimpanzees, anamnestic responses were observed that peaked near 10 mIU, a level that in humans is associated with protection from natural HBV infection. After HBV challenge of these chimpanzees, large anamnestic anti-HBs responses were observed, indicating HBV infections occurred in both animals. One chimpanzee experienced a mild case of hepatitis B and the other was protected from acute HBV-induced disease. In another HBV challenge study of chimpanzees immunized with a recombinant vaccinia virus expressing HBsAg (22), chimpanzees were primed immunologically to HBsAg following a single vaccination but did not develop detectable anti-HBs humoral responses. The chimpanzees were protected from acute clinical disease following HBV challenge but, as in the present study, were not protected from HBV infection.

Precise evaluation of the relative permissivity of chimpanzees and humans to enteric infection by Ad7 is not possible from the available data, as human trials for the Ad7 respiratory vaccine used vaccine dosages that were significantly lower than the dose of Ad7 used in this study. Nevertheless, analysis of the available data suggests that Ad7 replicates relatively well in the chimpanzee gut. Moreover, recombinant virus Wy-Ad7HZ6-1 replicated well in the chimpanzee gut as did Wy-Ad4HHxHS. The latter infections demonstrated the capacity of Ad4 to establish intestinal infection in chimpanzees.

The infection of chimpanzees by Wy-Ad7HZ6-1 is of particular interest, as this virus contains a large E3 deletion. The deletion extends from 80 to 84 m.u. and RNA analysis indicates (data not shown) that the major splice donor in E3 region is located at 80.2 m.u. Because the deletion removes this splice donor, essentially all known E3 region gene function is ablated. Although the functions of the E3 region gene products are, in general, poorly understood, it is known that the region is not essential for virus replication *in vitro*. Three of the approximately nine proteins encoded by E3 have been identified (23–25), and two of these proteins—14.7K and gp19K protein—have been implicated in protection of virus-infected cells from immune destruction (26–29). gp19K has been shown to mediate protection by blocking transport of class I major histocompatibility antigens to cell surfaces,

thus allowing virus-infected cells to avoid immune destruction by class I-restricted cytotoxic effector cells. The present study indicates that E3 gene functions are not required for sustaining enteric infections by Ad7. The presence of this region may thus rather be important for establishment of latent or persistent viral infections or perhaps for allowing reinfection of susceptible individuals.

A recent study of adenovirus mutants that have lost the ability to block expression of class I major histocompatibility antigens via deletion of sequences encoding gp19K has indicated that such mutants show increased capacity for induction of lung pathology and disease in cotton rats (30). In the present study, Wy-Ad7HZ6-1, which contains a deletion of the coding sequence for gp19K, did not cause enteric disease in chimpanzees.

With regard to the use of heterotypic vectors for booster applications, it should be noted that 42 antigenically distinct adenovirus serotypes have been described (31). A large proportion of these viruses have not been linked to disease states in humans and on that basis might be considered for use as vectors for vaccine applications. This diversity of serotypes could also be used to overcome problems of preexisting adenovirus immunity in vaccine recipients.

A seroprevalence study of chimpanzees at Sema indicated substantial preexisting immunity to Ad4 but not to Ad7. This antibody profile is consistent with both the known antigenic cross-reactivity between the chimpanzee adenovirus Pan 9 and human Ad4 as well as the demonstrated absence of antigenic relatedness between chimpanzee adenoviruses and human Ad7 (17). A similar seroprevalence study of chimpanzees housed at Buckshire (Perkasie, PA) indicated that 100% (10/10) of the animals tested were seropositive for Ad4 and 90% (9/10) were seropositive for Ad7 (unpublished data). The factors contributing to these differences in antibody profiles between chimpanzee groups are not known but may be related to differences in conditions under which the chimpanzees were maintained. Chimpanzees at Sema were housed in individual isolators, whereas the chimpanzees at Buckshire were not housed under isolator conditions. Isolation of the chimpanzees may have contributed to protection of the animals from contact with human adenoviruses or adenoviruses from other chimpanzees.

In summary, we have identified the chimpanzee as a model for testing Ad7- and Ad4-vectored vaccines and demonstrated the feasibility of using oral enteric vaccination with recombinant adenoviruses to induce humoral immune responses to foreign viral genes. This approach to vaccination may be adapted for immunization against many different diseases.

We thank F. Graupensperger, W. Magargle, B. Kostek, J. Barton, V. Jarocki-Witek, R. Mastroeni, and M. Shapiro for their skilled technical assistance and M. Hasson for manuscript preparation.

1. Tiollais, P., Pourcel, C. & Dejean, A. (1985) *Nature (London)* **317**, 489–495.
2. Ayoola, E. A., Balayan, M. S., Deinhardt, F., Gust, J., Kureshi, A. W., Maynard, J. E., Nayak, N. C., Schatzmayer, H. G. & Zhuang, H. (1988) *Bull. WHO* **66**, 443–455.
3. Top, F. H., Jr., Grossman, R. A., Bartelleni, P. J., Segal, H. E., Dudding, B. A., Russell, P. K. & Buescher, E. L. (1971) *J. Infect. Dis.* **124**, 148–154.
4. Top, F. H., Jr., Buescher, E. L., Bancroft, W. H. & Russell, P. K. (1971) *J. Infect. Dis.* **124**, 155–160.
5. Ballay, A., Levrero, M., Buendia, M.-A., Tiollais, P. & Perricaudet, M. (1985) *EMBO J.* **4**, 3861–3865.
6. Davis, A. R., Kostek, B., Mason, B. B., Hsiao, C. L., Morin, J., Dheer, S. K. & Hung, P. P. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7560–7564.
7. Morin, J. E., Lubeck, M. D., Barton, J. E., Conley, A. J., Davis, A. R. & Hung, P. P. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4626–4630.
8. Molnar-Kimber, K. L., Davis, A. R., Jarocki-Witek, V., Lubeck, M. D., Vernon, S. K., Conley, A. J. & Hung, P. P. (1987) in *Hepadna Viruses*, eds. Robinson, W., Koike, K. & Will, H. (Liss, New York), pp. 173–187.
9. Pacini, D. L., Dubovi, E. D. & Clyde, W. A., Jr. (1984) *J. Infect. Dis.* **150**, 92–97.
10. Hjorth, R. N., Bonde, G. M., Pierzchala, W. A., Vernon, S. K., Weiner, F. P., Levner, M. H., Lubeck, M.D. & Hung, P. P. (1988) *Arch. Virol.* **100**, 279–283.
11. Postlewaite, R. (1973) *Scott. Med. J.* **18**, 131.
12. Pereira, H. G. & Kelly, B. (1957) *Nature (London)* **157**, 615–616.
13. Pereira, H. G., Allison, A. C. & Niven, J. S. F. (1962) *Nature (London)* **196**, 244–245.
14. Rowe, W. P., Huebner, R. J., Hartley, J. W., Ward, T. G. & Parrott, R. H. (1955) *Am. J. Hyg.* **61**, 197–218.
15. Hayflick, L. & Moorhead, P. S. (1961) *Exp. Cell Res.* **25**, 585–621.
16. Cladaras, C. & Wold, W. S. M. (1985) *Virology* **140**, 28–43.
17. Willimzik, H.-F., Kalter, S. S., Lester, T. L. & Wigand, R. (1981) *Intervirology* **15**, 28–36.
18. Barker, L. F., Maynard, J. E., Purcell, R. H., Hoofnagle, J. H., Berquist, K. R., London, W. T., Gerety, R. J. & Krushak, D. H. (1975) *J. Infect. Dis.* **132**, 451–458.
19. Anderson, L. J., Godfrey, K., McIntosh, K. & Hierholzer, J. C. (1983) *J. Clin. Microbiol.* **18**, 463–468.
20. Maynard, J. E., Berquist, K. R., Krushak, D. H. & Purcell, R. H. (1972) *Nature (London)* **237**, 514–515.
21. McAuliffe, V. J., Purcell, R. H., Gerin, J. L. & Tyeryar, F. J. (1982) in *Viral Hepatitis: 1981 International Symposium*, eds. Szmuness, W., Alter, H. J. & Maynard, J. E. (Franklin Inst., Philadelphia), pp. 295–304.
22. Moss, B., Smith, G. L., Gerin, J. L. & Purcell, R. H. (1984) *Nature (London)* **311**, 67–69.
23. Persson, H., Jansson, M. & Phillipson, L. (1980) *J. Mol. Biol.* **136**, 375–394.
24. Tollefson, A. E. & Wold, W. S. M. (1988) *J. Virol.* **62**, 33–39.
25. Wold, W. S. M., Cladaras, C., Magie, S. C. & Yacoub, N. (1984) *J. Virol.* **52**, 307–313.
26. Gooding, L. R., Elmore, L. W., Tollefson, A. E., Brady, H. A. & Wold, W. S. M. (1988) *Cell* **53**, 341–346.
27. Andersson, M., Paabo, S., Nilsson, T. & Peterson, P. A. (1985) *Cell* **43**, 215–222.
28. Paabo, S., Bhat, B. M., Wold, W. S. M. & Peterson, P. A. (1987) *Cell* **50**, 311–317.
29. Burgert, H. G. & Kvist, S. (1985) *Cell* **41**, 987–997.
30. Ginsberg, H. S., Lundholm-Beauchamp, W., Horswood, R. L., Pernis, B., Wold, W. S. M., Chanock, R. M. & Prince, G. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3823–3827.
31. Wadell, G., Allard, A., Evander, M. & Li, Q.-C. (1986) *Chem. Scr.* **26B**, 325–335.