INFECTION AND IMMUNITY, March 1972, p. 300-304
Vol. 5, No. 3

Enteric Immunization with Live Adenovirus Type 21 Vaccine

II. Systemic and Local Immune Responses Following Immunization

ROBERT McNAIR SCOTT, BURTON A. DUDDING, SALVATORE V. ROMANO, AND PHILIP K. RUSSELL

Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, D.C. 20012

Received for publication 2 September 1971

Studies of the immunologic responses following administration of a live, enteric-coated adenovirus (ADV) type 21 vaccine showed that nine of ten vaccinees and none of five controls developed neutralizing antibody. Antibody activity of serum and secretory immunoglobulins was assayed by using a ^{14}C-labeled ADV-21 antigen in a radioimmunodiffusion system. Increases in immunoglobulin M, A and G (IgM, IgA, IgG) activity were detected in sera from vaccinees but not in those from controls. IgA copro antibody activity was also shown in vaccinees but not in controls. Nasal secretions showed no detectable IgA antibody responses by this method. These studies show marked differences in serum and local IgA antibody activity in induced enteric ADV infection compared to previously reported responses after natural infection. The protective role of secretory IgA in adenovirus infections is obscure. However, absence of nasal IgA responses may indicate that protection against disease with enteric ADV vaccines depends primarily upon humoral antibody.

It has been suggested that immunization procedures, to be effective, should simulate the immune responses induced by natural infection (3). In the case of viral upper respiratory disease, natural infection leads to the appearance of specific antibody both in serum and in the secretions at the local site of infection (4). Unnatural routes of infection with live viruses have been used to promote immunity against a number of diseases such as rubella, rubeola, and mumps. In the case of adenovirus, a respiratory pathogen, immunization against disease by using live enteric-coated virus preparations which promote asymptomatic intestinal infections has proved very effective (12). The mechanism by which immunity is induced by enteric adenovirus infections has not been investigated thoroughly. However, Smith et al. were unable to detect nasal neutralizing (N) antibody in volunteers immunized with adenovirus type 4 enteric vaccine who did develop serum N antibody (11). Since this same vaccine has been shown to be highly protective against adenovirus type 4-associated disease, it has been suggested that serum antibody alone affords protection against adenoviral disease.

The administration of adenovirus type 21 vaccine under controlled conditions (6) enabled us to re-examine the nasal responses to enteric immunization, to document the development of antibodies in the local secretions of the intestinal tract, and to examine in detail the development of immunoglobulins in the sera.

MATERIALS AND METHODS

Subjects. Immunologic responses after administration of adenovirus type 21 vaccine were investigated in the same 15 adult male volunteers in whom vaccine safety and efficacy were studied by Dudding et al. (6). Volunteers lacked detectable adenovirus type 21 N antibody at serum dilutions of 1:2. Capsules containing $10^{6.4}$ tissue culture infectious doses ($TCID_{50}$) of type 21 vaccine virus (V-270) were given to ten of the volunteers, and the remaining five received placebos. The men were housed in closed wards throughout the period of study.

Specimens. Samples of serum were obtained initially and at weekly intervals after immunization. Throat and nasal washes and stools were collected daily from 4 days before through 28 days after immunization. All samples were frozen immediately and stored at −70 C before being tested.

Nasal washes were obtained by irrigating the nasal mucosa with 10 ml of normal saline (1). Samples were mixed thoroughly with glass beads or sea sand and centrifuged at 12,000 × *g* for 20 min. The supernatant fraction was decanted and dialyzed against 200 volumes of distilled water. Protein concentrations were determined on the dialyzed samples with a Aminco-

Bowman spectrophotofluorimeter by fluorescence at 340 nm with an excitation wave length of 280 nm. The solutions were then lyophilized and reconstituted with distilled water to contain 250 mg of protein per 100 ml.

Stool samples were suspended in twice their weight of 0.9% saline and mixed with glass beads. Course particulate matter was pelleted by centrifugation at 750 × *g* for 20 min. The supernatant fluid was centrifuged at 12,000 × *g* for 0.5 hr. The resulting clarified solution was dialyzed overnight in 0.9% saline. After determination of volume, the specimens were lyophilized and reconstituted to the appropriate concentration with distilled water.

Sera were extracted with diethylaminoethyl (DEAE) Sephadex A-50 to isolate immunoglobulin G (IgG) by the method described by Altemeier et al. (1), with the modifications that the extracts were concentrated with Lyphogel (Gelman Instrument Co.).

Antisera to immunoglobulins. Monospecific antisera to IgG, and immunoglobulins M and A (IgM and IgA) were obtained commercially from Hyland Laboratories (lot numbers anti-IgA 8212M001A1, anti-IgG 8201H002A1, anti-IgM 8211M001A1). The antisera were shown to be specific by immunoelectrophoresis.

Quantitation of immunoglobulins. The immunoglobulins contained in sera, stools, and nasal washes were quantified by using a standard and low-level radial diffusion kits prepared by Hyland Laboratories.

Autoradiographic antigen preparation. ^{14}C-labeled adenovirus type 21 antigen was prepared in monolayer cultures of human embryonic kidney cells. Blake bottles containing monolayers were infected with adenovirus type 21 vaccine strain with a multiplicity of infection of approximately 10 to 1. After a 90-min incubation period, a maintenance medium, medium 199 containing 1% fetal bovine serum with one-tenth the normal amino acids, was added. To this was added 0.2 ml of a ^{14}C-amino acid mixture containing 0.168 mg of mixed L-amino acids and 0.1 mCi of ^{14}C per ml (New England Nuclear Corp.). The cultures were then incubated at 37 C. When cytopathology was noted in 75 to 100% of the cells, the medium was decanted, and the cells were harvested into 10 ml of Hank's balanced salt solution. The cells were disrupted sonically and centrifuged, and the supernatant fluid was treated twice with Genetron. The aqueous layer was recovered and dialyzed repeatedly against salt solution until the radioactivity in the dialysate was reduced to background level. The preparation used for radioactive binding studies contained 2.8×10^6 counts per min per ml and $10^{7.6}$ $TCID_{50}$ of adenovirus infective particles per ml. Dilutions of 1:10 or 1:100 of this antigen were used. Because all components of the virus were labeled, the antigen was broadly cross-reactive.

Radioimmune diffusion. Replicate micro-Ouchterlony plates were prepared with 1% agarose in 0.01 M tris (hydroxymethyl)aminomethane-ethylenediaminetetracetic acid buffer at *p*H 8.0. The peripheral wells were filled with twofold serial dilutions of the specimens to be tested. A specific antihuman immunoglobulin was then placed in the center well of each pattern. Twenty-four hours of incubation at room temperature was allowed for precipitin lines to develop, and the preparations were washed extensively with normal saline. The central wells were then fired with a dilution of the radioactive antigen. After a second 24-hr development period, the plates were washed extensively, dried, and placed in contact with Kodak X-ray film for 1 or 2 weeks. The slides were removed from the film and stained for protein with amido black, and the X-ray film was developed.

RESULTS

N and CF responses in sera. All nine of the ten immunized volunteers who excreted vaccine virus developed N antibodies by day 21 postimmunization. Antibody titers varied between 1:2 and 1:64. Individual and geometric mean N antibody titers at time after immunization are shown in Fig. 1. Infected vaccinees showed no significant increase in adenovirus complement-fixing (CF) antibody following immunization.

Radioimmune diffusion studies on sera. The chronological development of adenovirus antibody activity in the three major serum immunoglobulins was documented by specific radioimmune binding to immunoglobulin precipitin lines (Fig. 2). Initial sera from both the immunized and the control volunteers contained low levels of anti-adenovirus IgM and IgA. Rises in the anti-adenovirus activity of IgM were detectable by 1 week postimmunization. The IgM geometric mean titer peaked by the second week and fell slightly by the fourth week. IgA anti-adenovirus antibodies rose to a plateau by the third week. IgG from all subjects showed high initial binding. IgG, however, in contrast to IgM and IgA, showed no significant rises in geometric mean titers by this method; indeed, fourfold rises were found in only two of the nine immunized volunteers. Because of the high concentrations of group-reactive IgG antibody assayed by the radioimmunodiffusion method, small increases in

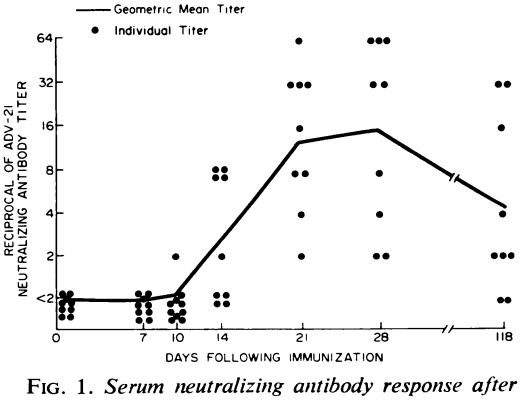

FIG. 1. *Serum neutralizing antibody response after administration of live enteric-coated adenovirus type 21 vaccine.*

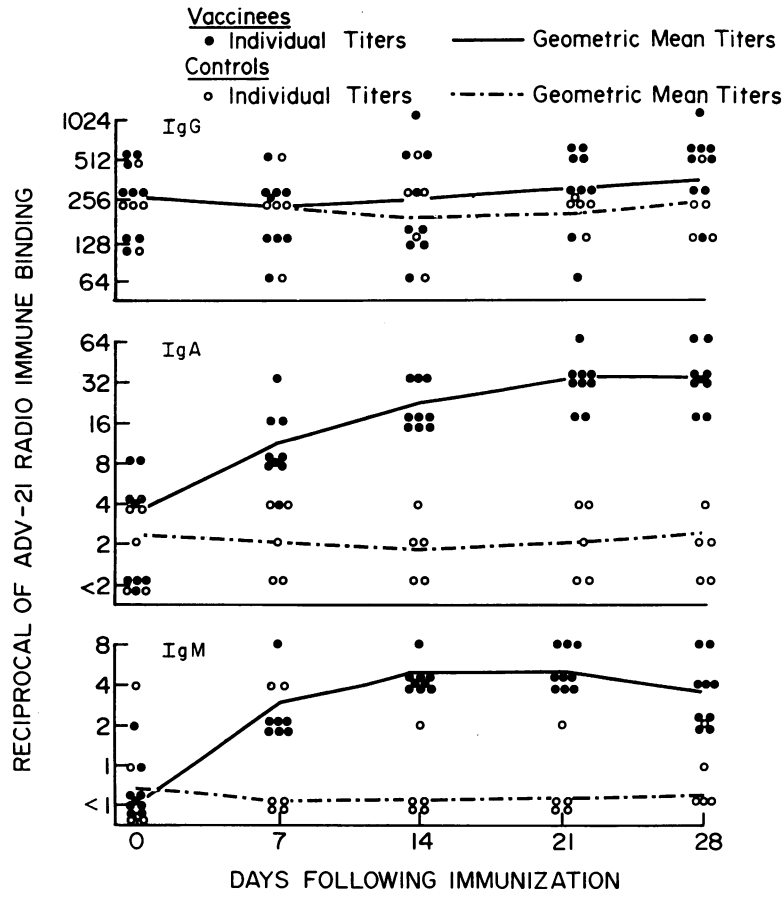

FIG. 2. *Serum radioimmune binding responses in the IgG, IgA, and IgM fractions after administration of live enteric-coated adenovirus type 21 vaccine.*

specific antibody might not be detectable. Therefore, IgG was extracted from the sera of several individuals by using DEAE Sephadex A-50. These extracts were concentrated to approximately the original amount of IgG and were found by immunoelectrophoresis to contain only IgG. Neutralization studies done on the whole sera and the extracted IgG from two volunteers showed parallel rises in neutralizing activity (Fig. 3).

Antibody responses of nasal secretions. Nasal washes were studied for the presence of adenovirus antibody. The relative amounts of IgG, IgM, and IgA were estimated in each concentrated specimen by using low-level immunoplates and serum standards. Table 1 shows that the relative amounts of IgA and the radioimmune diffusion titers on the IgA precipitates in the nasal washes were approximately the same on day 0 and day 28. Volunteer no. 10 is an exception, in that there was approximately twice as much IgA in the 28-day specimen as was present in the day 0 specimen. This volunteer developed a mild, afebrile upper respiratory illness apparently unrelated to the vaccination, and the rise in nasal IgA may be attributable to this. The volunteers who received placebo had similar relative IgA levels and radioimmune diffusion titers on early and late nasal washes.

Quantitative IgG levels obtained on nasal secretions ranged between less than 4 mg/100 ml to 15 mg/100 ml. Radioimmunodiffusion titers on IgG were present at levels of 1:2 to 1:4 but also showed no rises after immunization. No IgM was detected in any of the nasal secretions.

No N antibodies to adenovirus type 21 were detectable in nasal secretions taken on day 0 or day 28.

Antibody responses in the intestinal tract. As viral replication occurred in the lower alimentary tract, immune responses were looked for in fecal samples. Radioimmune binding of adenovirus by the copro IgA was demonstrated as early as the

second week in four of the immunized volunteers, and in seven of nine by the fourth week (Table 2). No other immunoglobulins were found in stools.

Fourfold concentrations of pooled stool samples from day −4 to 0 and day 25 to 28 were tested for neutralization activity in one immunized individual. Neutralizing activity was demonstrated only in the postimmunization sample.

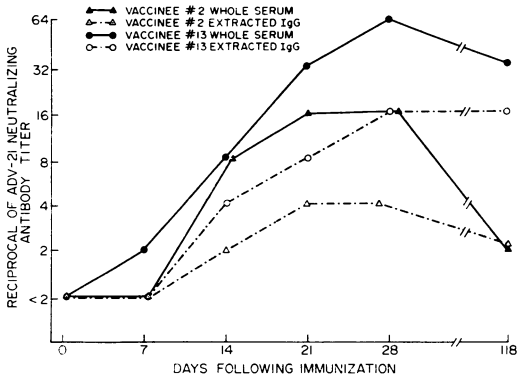

FIG. 3. *Neutralizing antibody responses in two vaccines in whole serum and DEAE-Sephadex-extracted IgG.*

TABLE 1. *Radioimmune binding of adenovirus antigen by nasal secretory immunoglobulin A (IgA)*

Subject	Nasal secretions[a]			
	IgA[b]		RID[c]	
	Day 0	Day 28	Day 0	Day 28
Immunized ADV-21 excretors				
1	38	31	2	<1
2	37	18	<1	<1
3	18	22	<1	<1
5	30	29	1	<1
6	31	45	<1	<1
8	16	19	4	4
10	15	33	2	4
11	34	32	1	1
13	39	42	<1	<1
Controls				
4	ND[d]	ND	1	<1
8	18	20	2	2
9	31	22	2	1
12	30	28	1	<1
14	33	33	1	1

[a] Nasal secretions adjusted to 250 mg of protein per 100 ml.

[b] Milligrams of IgA per 100 ml of nasal washes; versus serum standards.

[c] Reciprocal of radioimmune diffusion titer.

[d] Not done.

TABLE 2. *Radioimmune binding of adenovirus antigen by stool secretory immunoglobulin A (IgA)*

Subject	Stool IgA	
	Day 0	Day 2
Immunized ADV-21 excretors		
1	−	+
2	−	+
3	?	?
5	−	+
6	−	+
8	−	+
10	+	+
11	−	+
13	−	+
Controls		
4	−	−
7	−	−
9	+	+
12	−	−
14	−	−

Two subjects, one in the control group and one in the immunized group, were found to have preexisting IgA adenovirus antibody in the stool. In another immunized individual, the antibody assays were inconclusive due to insufficient IgA. No adenovirus binding was found in stools from any of the four remaining control volunteers.

DISCUSSION

Enteric infection with adenovirus type 21 causes serum antibody responses similar to those reported after local (selective) intestinal infection with adenovirus types 4 and 7. All three enteric vaccines commonly result in low levels of N antibody and poor CF antibody responses following immunization (5, 7). These findings contrast sharply with immune responses after naturally occurring upper respiratory tract infections with these adenoviruses which characteristically result in high N and CF antibody responses (13).

By using a radioimmune diffusion technique similar to the technique used in this study, as well as serum separation methods, Bellanti reported the immunochronology of 23 patients after natural upper respiratory infection with adenovirus type 4 (3). IgM antibodies developed in all but 2 of 23 patients tested, and IgA developed in 16 of 17 tested by radioimmunodiffusion. Neither antibody class was detected in preinfection sera. IgG was detected in many preinfection sera, reflecting both the broadly cross-reactive properties of the antigen used and the prior exposure of the

individuals to adenoviruses. However, marked IgG responses were documented following natural infection in these individuals.

Vaccine-induced enteric adenovirus-21 infection induced levels of IgM and IgA antibodies similar to those following natural infection. However, in our studies, many individuals had detectable IgM and IgA antibodies in preinfection sera, reflecting again the use of a broadly cross-reactive adenovirus antigen and the presumption that many individuals had had recent adenovirus infections. In contrast to natural infection accompanied by large increases in specific IgG antibody, increases in the IgG class after enteric immunization were not detectable by radioimmune diffusion. That adenovirus-21-specific IgG antibodies did develop in these individuals was demonstrated in DEAE-Sephadex-IgG extracts tested in an adenovirus type 21 neutralization system.

Nasal secretory adenovirus antibody responses were not detected in any of the immunized volunteers in this study. This supports the observation of Smith et al. that nasal antibody was not produced following immunization with adenovirus type 4 enteric vaccine and contrasts sharply with Bellanti's study which showed that naturally occurring adenovirus infections were uniformly accompanied by development of nasal secretory antibody (3, 11). Secretory IgA adenovirus antibody did develop in the intestinal tracts of the majority of immunized individuals in this study. This substantiates the finding of Ogra et al. that local stimulation by antigen is necessary for the production of local antibody (10).

The fact that enteric adenovirus infections afford protection without inducing detectable nasal secretory antibody suggests that a different protective mechanism exists than that which has been postulated for other upper respiratory virus infections such as rhinoviruses, respiratory syncytial virus, or para-influenza viruses. In the latter infections, local respiratory IgA antibody is required for protection against disease (4).

After natural infection with type 4 adenovirus, reinfection rarely occurs. With enteric immunization, on the other hand, reinfection of the respiratory tract may occur with viral shedding and the development of local nasal antibody. Clinically, this reinfection may be asymptomatic or mild (11). Disease associated with reinfection, as defined by temperature elevation and systemic signs, rarely occurs. Invasiveness beyond the mucosa, where serum IgG may be important in protection, therefore, may be important in the pathogenesis of febrile adenovirus disease. Viremia and viruria are known to occur in individuals hospitalized with febrile adenovirus disease (8) but are rare in para-influenza or rhinovirus infections. This, coupled with the observations that parenterally administered live adenovirus experimental vaccines cause febrile disease (9) further suggests that viremia or extra-respiratory replication, or both, occur. Thus, it seems probable that the typical febrile disease associated with natural adenovirus infection is prevented by the presence of serum N antibody. Secretory antibody may be of importance only in the prevention of local infection.

LITERATURE CITED

1. Altemeier, W. A. III, F. K. Mundon, F. H. Top, Jr., and P. K. Russell. 1970. Method for extracting viral hemagglutination-inhibiting antibodies from nonspecific inhibitors of serum. Appl. Microbiol. **19**:785–790.
2. Artenstein, M. S., J. A. Bellanti, and E. L. Buescher. 1964. Identification of antiviral substances in nasal secretions. Proc. Soc. Exp. Biol. Med. **117**:558–564.
3. Bellanti, J. A., A. S. Artenstein, B. L. Brandt, B. S. Klutinis, and E. L. Buescher. 1969. Immunoglobulin responses in serum and nasal secretions after natural adenovirus infections. J. Immunol. **103**:891–898.
4. Chanock, R. M. 1970. Control of acute mycoplasmal and viral respiratory tract disease. Science **169**:248–256.
5. Couch, R. B., R. M. Chanock, T. R. Cate, D. S. Lang, V. Knight, and R. J. Huebner. 1963. Immunization with types 4 and 7 adenovirus by secretive infection of the intestinal tract. Amer. Rev. Resp. Dis. **88**:394–403.
6. Dudding, B. A., P. J. Bartelloni, R. M. Scott, F. H. Top, Jr., P. K. Russell, and E. L. Buescher. 1971. Enteric immunization with live adenovirus type 21 vaccine. I. Tests for safety, infectivity, immunogenicity, and potency in volunteers. Infect. Immunity **5**:295–299.
7. Edmondson, W. P., R. H. Purcell, B. F. Gundelfinger, J. W. P. Love, W. Ludwig, and R. M. Chanock. 1966. Immunization by selective infection with adenovirus grown in human diploid tissue culture. J. Amer. Med. Ass. **195**:453–459.
8. Guntekust, R. R., and A. D. Heggie. 1961. Viremia and viruria in adenovirus infections. N. Engl. J. Med. **261**:374–378.
9. Hilleman, M. R., R. E. Hodges, M. S. Warfield, and S. A. Anderson. 1957. Acute respiratory illness in volunteers following intramuscular administration of live adenovirus. J. Clin. Invest. **36**:1072–1080.
10. Ogra, P. L., and D. T. Karzon. 1969. Distribution of poliovirus antibody in serum, nasopharynx and alimentary tract following segmental immunization of lower alimentary tract with poliovirus. J. Immunol. **102**:1423–1430.
11. Smith, T. J., E. L. Buescher, F. H. Top, Jr., W. A. Altemeier, and J. M. McCown. 1970. Experimental respiratory infection with type 4 adenovirus vaccine in volunteers: clinical and immunological responses. J. Infec. Dis. **122**:239–248.
12. Top, F. J., B. A. Dudding, P. K. Russell, and E. L. Buescher, 1971. Control of respiratory disease in recruits with type 4 and 7 adenovirus vaccines. Amer. J. Epidemiol. **94**:142–146.
13. Van Der Veen, J., K. G. Oei, and M. F. W. Abarbanel. 1969. Patterns of infections with adenovirus types 4, 7 and 21 in military recruits during a 9-year survey. J. Hyg. **67**:255–268.