INFECTION AND IMMUNITY, Feb. 1976, p. 354-359

Vol. 13, No. 2
Printed in U.S.A.

Antigenic Characterization of Intermediate Adenovirus 14-11 Strains Associated with Upper Respiratory Illness in a Military Camp

JOHN C. HIERHOLZER* AND AGUSTIN PUMAROLA

Respiratory Virology Branch, Bureau of Laboratories, Center for Disease Control, Atlanta, Georgia 30333, and Department of Virology, Faculty of Medicine, University of Barcelona, Barcelona, Spain*

Received for publication 29 August 1975

An unusual variant of adenovirus (AV) 11 was isolated from throat and rectal swabs from six persons with upper respiratory illness in a Spanish military camp in March 1969. The same strain was serologically related to the upper respiratory illness of seven other men among 25 sample cases studied in detail. After strain purification, the virus was grouped as an AV by standard biological tests; it possessed the usual titers of group-specific hexon antigen but only low hemagglutinin titers (1:4 to 1:8) with erythrocytes from selected rhesus monkeys. The virus gave little reaction in hemagglutination inhibition (HI) tests with antisera to AV 1 through 35, but was neutralized to homologous titers by AV 11 antiserum. Reciprocally, rabbit and guinea pig antisera to the isolates possessed high HI antibody titers to prototype AV 14 and high serum neutralization (SN) antibody titers to prototype AV 11. On this basis, the variants were classified as AV 14-11 intermediates. Sequential serum specimens from the patients with and without positive cultures showed diagnostic rises in HI and SN antibody levels to the AV 14-11 intermediate and to prototype AV 11, but little response to AV 14.

In March 1969, an outbreak of upper respiratory illness occurred among new recruits at the San Clemente Sasebas military camp in Gerona, Spain. Among a randomly selected group of cases studied in detail, influenza A and adenovirus (AV) types 3, 7a, and 11 were involved to varying extents; however, AV 11 was the predominant etiologic agent (9). Initial antigenic characterization of the influenza A and AV 3 and 7a isolates was unremarkable in that these were typical or currently endemic strains. In contrast, the AV 11 isolates were uniformly atypical, because they lacked readily detectable hemagglutinins and were identifiable only by neutralization tests (9). An extensive study of these atypical AV 11 isolates was then initiated to determine whether they were intermediate strains and whether the presence of multiple viruses in the upper respiratory illness outbreak somehow effected the origin of these strains.

MATERIALS AND METHODS

Strain purification. Virus strains 266 and 273 were originally recovered in human epidermoid carcinoma (HEp-2) cell cultures (9). The strains were passaged thenceforth only in HEp-2 and primary human embryonic kidney (HEK) cells. Both viruses were strain purified by consecutive triple terminal dilution passages in HEK, followed by triple end point dilution plaque purifications in HEp-2, as previously described (5).

Sterility checks on stock passages were performed by inoculating bacteriological, mycological, and mycoplasmal media to reveal nonviral contaminants (8), by breakthrough neutralization tests to detect other complete viruses (3, 6), and by complement fixation (CF) tests (1, 12) and electron microscopy to reveal AV-associated virus types 1 to 4 (5, 12, 14). Antisera to the strain-purified viruses were prepared in New Zealand white rabbits and Hartley strain guinea pigs by three biweekly subcutaneous injections of virus in Freund incomplete adjuvant, with exsanguination 2 weeks after the last injection (6).

Virus characterization. Classical virus characterization procedures were used to place the strains in the proper group (13, 15, 22). Nucleic acid type, acridine orange staining, chloroform and acid stability, and temperature stability with or without 1 M $MgCl_2$ were determined as described (5). Electron microscopy was carried out by pseudoreplica examination of concentrated supernatant fluids from infected HEK cells (5). Microculture slides of human embryonic lung fibroblast cells infected with virus until 3^+ to 4^+ cytopathology appeared were stained for viral inclusions by the May-Grünwald-Giemsa and the Van Orden inclusion stains (5).

The group CF antigen (hexon) was assayed by block titration against anti-AV 2 hexon mouse immune ascitic fluid in the standardized CF test with

overnight fixation of 5 U of complement (1, 2). Hemagglutinating antigens were assayed by the standardized hemagglutination (HA) and hemagglutination inhibition (HI) tests with 0.01 M phosphate-buffered saline diluent, pH 7.2, and 0.4% mammalian or 0.5% avian erythrocyte suspensions (10, 11). HA tests were performed with a variety of mammalian and avian species at 37 and 2 C to attempt precise subgrouping of the viruses (4). HI tests were performed with virus against reference equine antisera to AV 1 to 33 (7) and with prototype AV 1 to 33 viruses against rabbit and guinea pig antisera to strains 266 and 273. Serum neutralization (SN) tests were carried out with the same reagents by the 3-day test in primary monkey kidney cell cultures (20).

RESULTS

The epidemiology of the upper respiratory illness outbreak, during which these atypical strains were isolated, has been described in an earlier report (9). For 13 of the 25 cases studied in detail, the etiologic agent was AV "11" alone (nine cases) or AV "11" followed by influenza A [A/Hong Kong/8/68(H3N2)], which was endemic in Spain and most of Europe in 1969 (17, 18). The clinical features, sources of virus isolation, and serological confirmation of infection in these cases are presented in Table 1. AV "11" was isolated in HEp-2 cell cultures from throat swabs from six of eight patients (75%) between 2 and 5 days after onset of clinical illness. The virus was also recovered from the rectal swab taken 6 days after onset from one of these six patients. The same strain apparently infected the two patients with negative cultures and five from whom no cultures were obtained, because HI and SN tests with selected AV "11" isolates and with acute and convalescent sera from all 13 patients revealed serological responses consistently similar within the entire group.

On initial testing, the AV "11" isolates replicated to high infectivity titers in tissue cultures of human origin but failed to agglutinate erythrocytes of rhesus or vervet monkeys, humans, rats, guinea pigs, dogs, chickens, geese, or turkeys at 37 or 2 C. Growth characteristics and cytopathology were indicative of Rosen's HA group 1 viruses (19), and CF tests with antisera to the AV 2 hexon antigen (2), herpes simplex virus, and respiratory syncytial virus confirmed the isolates to be adenoviruses. SN tests with antisera to AV 12, 18, 20, 25, 28, 29, and 31 were negative, but SN tests with antisera to the group 1 viruses readily identified the isolates as AV 11. All isolates were neutralized to the homologous serum titer (1:160) of the reference equine antiserum to AV 11 (7) and exhibited no SN cross-reaction with AV 3, 7a, 14, 16, or 21 antisera.

Two of the strains were then selected for further studies: 266, from patient 12 (Table 1), with date of onset 15 March 1969 and 273, from patient 11, with onset 14 March 1969. These isolates were strain purified in HEK and HEp-2

TABLE 1. *Clinical and laboratory findings from 13 patients with upper respiratory illness associated with an AV 14-11 intermediate strain*

Patient	Age	Sex	Clinical findings		Virus isolated from[a]		Serological identification of infection[b]
			Maximum temp (C)	Signs/symptoms	TS	RS	
1	21	M	38	Myalgia	Negative (3)	–[c]	AV 11, influenza A
2	21	M	38	Pharyngitis, vomiting	AV 11 (3)	–	AV 11
3	21	M	38.5	Pharyngitis, chills, cough	AV 11 (3)	AV 11 (6)	AV 11
4	22	M	37	Pharyngitis	–	–	AV 11
5	22	M	39	Headache, myalgia, cough	–	–	AV 11, influenza A
6	21	M	38.7	Pharyngitis	AV 11 (3)	–	AV 11, influenza A
7	22	M	38	Pharyngitis	Negative (4)	–	AV 11
8	21	M	37	Pharyngitis	–	–	AV 11
9	21	M	37	Tonsillitis	–	–	AV 11
10	21	M	39	Pharyngitis, myalgia	–	–	AV 11
11	20	M	40	Pleurodynia, cough	AV 11 (3)	–	AV 11, influenza A
12	22	M	37.5	Pharyngitis, cough	AV 11 (2)	–	AV 11
13	21	M	38.5	Pharyngitis	AV 11 (5)	–	AV 11

[a] TS, Throat swab; RS, rectal swab. Numbers in parentheses are days after onset of clinical illness; the AV 11 identified by SN test is the 14-11 strain.

[b] Serological identification of infection as determined by a fourfold or greater rise in serum antibody titer in CF, HI, and/or SN tests.

[c] –, No specimen collected.

cells. Stock passages of the purified viruses were free of bacterial, fungal, and mycoplasmal contaminants detectable in the systems used and were free of other complete viruses as determined by SN breakthrough tests. CF tests for AV-associated virus 1 to 4 were negative, as were HA tests with human "O" cells at 4 C for AV-associated virus 4 (14) and electron microscopic searches for AV-associated virus.

The viruses were inhibited by iododeoxyuridine and produced yellow-green nuclear fluorescence with acridine orange stain, findings consistent with double-stranded deoxyribonucleic acid viruses. The viruses were chloroform stable (5% $CHCl_3$, 10 min), acid stable (pH 3.0, 4 h), and heat labile (50 C, 1 h) at pH 7.0, with no cationic stabilization.

Electron microscopy of the viruses in negatively stained pseudoreplica preparations from clarified virus culture fluids revealed typical AV morphology. Virion diameters averaged 74 nm excluding the fiber projections. Hexon and vertex capsomeres and the fibers were clearly discernible on the virion; no envelope or limiting membrane was present.

The viruses produced Cowdry type B basophilic intranuclear inclusions in human embryonic lung fibroblast microcultures. They replicated only in tissue cultures of human origin and produced 10^3 to 10^7 median tissue culture infectious doses of virus per 0.1 ml at 7 to 14 days and with typical AV cytopathology. They replicated in monkey kidney cell cultures only if potentiated by a helper virus such as simian virus 40. The viruses were not pathogenic for embryonated eggs or suckling mice, even after several passages by multiple routes of inoculation.

Supernatant fluids from virus-infected cells, harvested 2 days after complete cytopathology, contained soluble CF and HA antigens characteristic of the AV group. In CF block titrations these cultures had antigen titers of 1:16 against 1:256 titers (optimal dilutions) of AV 2 hexon mouse immune ascitic fluid, but no titer with antisera to herpes simplex or respiratory syncytial viruses. HA tests with erythrocytes from human O cells, rats, mice, gerbils, guinea pigs, dogs, chickens, geese, turkeys, cows, and sheep were consistently negative at 37 and 2 C (4). HA tests with erythrocytes from 44 different rhesus and vervet monkeys were essentially negative, with erythrocytes from only two rhesus monkeys giving HA titers at 37 C as high as 1:4 or 1:8. The erythrocytes from these same two monkeys were agglutinated with prototype AV 11 to 1:8,192 titers and with prototype AV 14 to 1:512 titers, indicating that these were exceptionally sensitive erythrocytes.

Antigenic characterization of the strain-purified isolates was carried out by complete reciprocal HI and SN testing. Rabbit and guinea pig antisera to the isolates were used with reference equine antisera to AV 1 to 33 (7) and rabbit antisera to candidate AV types 34 (5) and 35 (16) to determine interrelationships among prototype AV 1 to 35 strains and the AV "11" strains. The results are shown in Table 2. The virus strains are clearly identifiable with prototype AV 11 antisera by SN tests but not by HI tests. Reciprocally, the strain-specific antisera suggest that the strains are AV 14 by HI tests and AV 11 by SN tests. Only rabbit antisera data are shown in Table 2, but guinea pig antisera, prepared with the same immunizing antigens, had HI and SN titers identical to those for the rabbit sera. We therefore classify these AV "11" isolates as AV 14-11 intermediate strains according to the terminology of Wigand and Fliedner (21). That is, the strains would be considered AV 14 by HI but AV 11 by SN tests. Unilateral or bilateral cross-reactions between the AV 14-11 strains and AV 7a, 11, 14, 21, and 35 occur at titers of 1:5 to 1:20 in the HI test, and cross-reactions between the intermediates and AV 14 and 35 occur at titers of 1:5 to 1:10 in the SN test.

Acute (S-1), convalescent (S-2), and late convalescent (S-3) sera from all 13 AV "11" patients were tested by HI and SN against the prototype strains of AV 3, 7a, 11, 14, 16 and 21 and against representative strains of AV 3, 7a, and 14-11 isolated during this outbreak. The data on the six patients with positive AV 14-11 isolations are representative of the 13 and are shown in Table 3. The CF antibody response to the group-specific hexon antigen observed in all patients was typical of AV infections. Prior experience with AV 3 and 7a was evident in seven patients (five shown in Table 3) and with AV 7a alone in three patients, with current AV 14-11 infection giving significant heterotypic anamnestic responses to these types. The HI and SN antibody titers of the patients' sera to prototypes AV 3 (G.B.) and 7a (S-1058) were identical to those obtained with representative strains from this outbreak (274 and 265, respectively). Prototype AV 11 (Slobitski) virus gave HI and SN serum titers which were similar but somewhat lower than those obtained with a representative AV 14-11 isolate (273) (Table 3). Antibodies to prototype AV 14 (deWit) were not detected in any patient's sera by HI test but were detected to low levels by the SN test. Hence, by both HI and SN tests, all six patients with positive cultures of AV 14-11 intermediate strain, as well as the seven AV "11" patients with negative or no cultures, responded sero-

TABLE 2. *Relationship of intermediate AV 14-11 isolates 266 and 273 to the prototype strains of AV 1 through 35*

Adenovirus type	Antiserum[a] to AV type:								
						Candidate		Spain URI	
	1-6, 8-10, 12-13, 15-20, 22-33	7a	11	14	21	34	35	266	273
HI titers									
1-6, 8-10, 12-13, 15-20, 22-33	—[b]	—	—	—	—	0[c]	0	0	0
7a	—	160[d]	20	10	0	0	0	10	0
11	—	10	160	10	10	0	0	20	10
14	—	20	10	40	10	0	10	160	80
21	—	0	0	0	40	0	0	0	0
34	0	0	0	0	5	320	160	0	0
35	0	0	5	0	5	20	640	0	0
266	0	10	20	10	10	0	10	160	40
273	0	10	20	10	10	0	10	160	40
SN titers									
1-10, 12-13,	—	—	—	—	—	0	0	0	0
11	—	10	160	20	0	0	20	320	160
14	—	10	10	160	0	0	0	10	0
34	0	0	0	0	0	640	0	0	0
35	0	0	5	0	0	0	320	10	10
266	0	0	160	0	0	0	5	320	160
273	0	0	160	0	0	0	10	320	160

[a] Reference equine antisera to AV 1-33; rabbit antisera to AV 34, 35, Spain 266, and Spain 273. URI, Upper respiratory illness.

[b] For homologous and heterologous HI and SN titers, see reference 7.

[c] 0 = <5.

[d] For definitions of titers, see Table 3, footnotes *b* and *c*.

logically with high antibody titers to the homotypic virus, with slightly lower titers to prototype AV 11 and little or no detectable titer to prototype AV 14.

DISCUSSION

The atypical AV "11" strains isolated during this upper respiratory illness outbreak were clearly human AV by classical virus grouping tests and were typed as AV 11 by neutralization tests only. The strains possessed virtually no hemagglutinating activity and were unidentifiable by HI tests. Rabbit and guinea pig antisera prepared against strain-purified viruses contained high HI antibody titers to AV 14 and high SN antibody titers to AV 11, which indicated that the viruses were AV 14-11 intermediate strains. Human convalescent sera from this outbreak, on the other hand, contained high HI and SN antibodies to AV 11 and the AV 14-11 strain but no HI and little SN antibody to AV 14, which suggested that the intermediate was antigenically more closely related to AV 11 than to AV 14.

The clear and consistent discrepancy between antibody titers in the animal antisera (Table 2) and those in the human sera (Table 3) is difficult to explain. The test results were always repeatable and were similar for the four rabbits and guinea pigs (as one set of data) and for the 13 patients (as another set of data). The laboratory animals were immunized by subcutaneous injections of virus emulsified in adjuvant; they experienced no natural infection. The patients, however, experienced natural infection not only with AV 14-11 but earlier with antigenically related viruses (AV 3 and 7a) as well. Undoubtedly, the immune response is different for these groups, and this may account for the differing patterns of HI and SN data. In any event, the discrepancies (namely, no HI titer to AV 14 in human sera but high titers in animal sera; low but definite SN titers to AV 14 in human sera but no titers in animal sera; and

TABLE 3. *Homotypic and heterotypic antibody responses to group 1 AV after natural infection with the AV 14-11 intermediate strain*

Patient	AV 14-11 strain isolated	Serum sample (days after onset)	Patients' serum antibody titers								
			CF test[a] (AV 2 hexon)	HI test[b]					SN test[c]		
				AV 3 (274)	AV 7a (265)	AV 14-11 (273)	AV 11 (Slobitski)	AV 14, 16, 21 (prototypes)	AV 14-11 (273)	AV 11 (Slobitski)	AV 14 (deWit)
2	272	S-1 (3)	<8	16	16	<8	<8	<8	<8	<8	<8
		S-2 (17)	32	64	32	128	64	<8	16	16	8
		S-3 (28)	16	64	32	512	128	<8	32	32	16
3	271, 295	S-1 (3)	8	8	8	<8	<8	<8	<8	<8	<8
		S-2 (17)	16	16	32	64	16	<8	32	16	8
		S-3 (28)	32	16	64	256	128	<8	128	32	16
6	263	S-1 (3)	<8	<8	<8	<8	<8	<8	<8	<8	<8
		S-2 (17)	64	<8	<8	32	8	<8	16	8	<8
		S-3 (28)	32	<8	<8	32	32	<8	128	64	8
11	273	S-1 (3)	8	32	16	8	8	<8	<8	<8	<8
		S-2 (17)	64	256	32	256	128	<8	512	256	8
		S-3 (28)	64	256	32	256	128	<8	512	256	8
12	266	S-1 (2)	<8	32	8	<8	<8	<8	<8	<8	<8
		S-2 (16)	128	128	32	64	64	<8	32	16	8
		S-3 (27)	32	128	32	512	256	<8	64	32	16
13	270	S-1 (5)	<8	8	8	<8	<8	<8	<8	<8	<8
		S-2 (19)	64	512	128	512	128	<8	512	256	16
		S-3 (30)	32	256	32	512	256	<8	512	256	16

[a] Purified AV 2 hexon (2) measuring in this test strictly the AV group specificity. Titers are listed as dilution factor of optimal dilution of antibody in patient's sera when tested with optimal dilution of hexon antigen.

[b] HI antibody titers are listed as dilution factor of the highest dilution of serum completely inhibiting 4 HA units of virus per 0.025 ml in 1 h at 37 C. AV 3 strain 274, AV 7a strain 265, and AV 14-11 strain 273 were isolated during this outbreak; other test antigens were prototype strains.

[c] SN antibody titers are listed as dilution factor of the highest dilution of serum effecting a 2^+ reduction in cytopathology in monkey kidney cells from that observed in the working dilution (virus control) in 3 days.

comparatively high HI titers to AV 11 in human sera but low titers in animal sera) emphasize an inherent difficulty in defining hybrid strains. Conceivably, an intermediate strain could be defined one way if only data from immunized animals are used and quite differently if only data from convalesced patients are used. This problem requires further study.

Most patients whose illness was etiologically related to AV 14-11 already possessed high levels of antibody to AV 3 and 7a but not to the remaining group 1 AV types. Whereas this undoubtedly influenced the spread of the AV 14-11 strain in the outbreak, the total absence of HI and SN antibody to AV 11 and 14 in the acute sera indicated that prior experience of the patients with AV 11 or 14 probably played no role or exerted no biological pressure in the origin of the AV 14-11 intermediate strain. The development of such a strain thus remains a fascinating question, in contrast to the ever evolving strains of influenza clearly pressured by prior antibody experience in the host population. Perhaps the continuing study of intermediate strain AV in relation to their related serotypes will shed some light on their origin and demise.

ACKNOWLEDGMENTS

We appreciate the critical review of this study by Walter R. Dowdle, and the technical assistance of Patricia Bingham, Katharine Hilliard, Carol Reed, and Wilma Yarbrough.

LITERATURE CITED

1. Casey, H. L. 1965. Standardized diagnostic complement fixation method and adaptation to micro test. *In* Public Health Service, Monogr. no. 74, Washington, D.C.
2. Dowdle, W. R., M. Lambriex, and J. C. Hierholzer. 1971. Production and evaluation of a purified adenovirus group-specific (hexon) antigen for use in the diagnostic complement fixation test. Appl. Microbiol. 21:718-722.
3. Hampil, B., and J. L. Melnick. 1969. Method for testing virus stocks for viral contaminants. Appl. Microbiol. 17:17-20.
4. Hierholzer, J. C. 1973. Further subgrouping of the human adenoviruses by differential hemagglutination. J. Infect. Dis. 128:541-550.
5. Hierholzer, J. C., N. O. Atuk, and J. M. Gwaltney. 1975. New human adenovirus isolated from a renal transplant recipient: description and characterization of candidate adenovirus type 34. J. Clin. Microbiol. 1:366-376.

6. Hierholzer, J. C., and W. R. Dowdle. 1970. Hemagglutination properties of adenovirus types 20, 25, and 28. Proc. Soc. Exp. Biol. Med. 134:482-488.
7. Hierholzer, J. C., W. C. Gamble, and W. R. Dowdle. 1975. Reference equine antisera to 33 human adenovirus types: homologous and heterologous titers. J. Clin. Microbiol. 1:65-74.
8. Hierholzer, J. C., W. C. Gamble, K. D. Quist, and W. A. Chappell. 1972. Comparison of immunization methods for producing reference adenovirus antisera in horses. Appl. Microbiol. 24:398-404.
9. Hierholzer, J. C., A. Pumarola, A. Rodriguez-Torres, and M. Beltran. 1974. Occurrence of respiratory illness due to an atypical strain of adenovirus type 11 during a large outbreak in Spanish military recruits. Am. J. Epidemiol. 99:434-442.
10. Hierholzer, J. C., and M. T. Suggs. 1969. Standardized viral hemagglutination and hemagglutination-inhibition tests. I. Standardization of erythrocyte suspensions. Appl. Microbiol. 18:816-823.
11. Hierholzer, J. C., M. T. Suggs, and E. C. Hall. 1969. Standardized viral hemagglutination and hemagglutination-inhibition tests. II. Description and statistical evaluation. Appl. Microbiol. 18:824-833.
12. Hoggan, M. D., N. R. Blacklow, and W. P. Rowe. 1966. Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics. Proc. Natl. Acad. Sci. U.S.A. 55:1467-1474.
13. Hsiung, G. D. 1973. Diagnostic virology - an illustrated handbook, 2nd ed. Yale University, New Haven, Conn.
14. Ito, M., and H. D. Mayor. 1968. Hemagglutinin of type 4 adeno-associated satellite virus. J. Immunol. 100:61-68.
15. Lennette, E. H., and N. J. Schmidt (ed.). 1969. Diagnostic procedures for viral and rickettsial infections, 4th ed. American Public Health Association, Inc., New York.
16. Myerowitz, R. L., H. Stalder, M. N. Oxman, M. J. Levin, M. Moore, J. D. Leith, N. H. Gantz, J. Pellegrini, and J. C. Hierholzer. 1975. Fatal disseminated adenovirus infection in the recipient of a renal transplant. Am. J. Med. 59:591-598.
17. Pumarola, A. 1969. Influenza (general discussion). Bull. W.H.O. 41:407-408.
18. Pumarola, A., A. Rodriguez-Torres, and M. Beltran. 1970. La gripe Hong-Kong en Barcelona. (In Spanish). Med. Trop. (Madrid) 4:221-250.
19. Rosen, L. 1960. A hemagglutination-inhibition technique for typing adenoviruses. Am. J. Hyg. 71:120-128.
20. Stevens, D. A., M. Schaeffer, J. P. Fox, C. D. Brandt, and M. Romano. 1967. Standardization and certification of reference antigens and antisera for 30 human adenovirus serotypes. Am. J. Epidemiol. 86:617-633.
21. Wigand, R., and D. Fliedner. 1968. Serologically intermediate adenovirus strains: a regular feature of Group II adenoviruses. Arch. Gesamte Virusforsch. 24:245-256.
22. Wilner, B. I. 1969. A classification of the major groups of human and other animal viruses, 4th ed. Burgess Publishing Co., Minneapolis.