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Recent Adenovirus Isolates Exhibiting Broad Intratypic and Intertypic Antigenicity.* (32130)

WADE P. PARKS, LAURA M. QUEIROGA, JOSEPH L. MELNICK, AND HELIO G. PEREIRA
World Health Organization International Reference Centre for Enteroviruses, Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas and World Health Organization International Influenza Centre, National Institute for Medical Research, Mill Hill, London, England

Typing of human adenovirus isolates is routinely accomplished by demonstrating a serological relationship with one of the prototype viruses using either serum neutralization (Nt) or hemagglutination-inhibition (HI), or preferably both(1-3). Recent reports have described strains which demonstrate intertypic crossings when tested by either technique or by both(4-10). At present, the significance of this antigenic overlap and variation within the adenovirus group is not clear, but practically it poses important problems for investigators concerned with the identification of field isolates. In a recent study of infantile diarrhea(11), several adenovirus strains were recovered which behaved as variants of prototype strains. The present report describes those strains which were found to be serologically related to, but certainly not identical with, prototype strains, and further demonstrates the importance of the reactivity of the antisera used in typing adenovirus isolates.

Materials and methods. Viruses and sera. Prototype human adenovirus types 1-18 were received from Dr. W. Dowdle, Communicable Disease Center (CDC), Atlanta, Georgia, who also kindly performed some serological tests with the strains described in this report. Additional strains of type 14 were obtained from Dr. M. Green, St. Louis and Dr. R. Chanock, Bethesda. Types 19-30 were obtained from the American Type Culture Collection. The isolates referred to as Karachi strains were recovered from rectal swabs collected during a study of infantile diarrhea in Pakistan(11). Isolates were propagated in monolayer cultures of human fetal kidney cells maintained with Eagle's medium containing 2% fetal bovine serum and 0.225% sodium bicarbonate. For hemagglutination studies, virus stocks were prepared in KB monolayer cell cultures maintained with medium 199 containing inactivated chicken serum and 0.225% sodium bicarbonate. Twenty-four hours after the infection had detached all the cells from the glass (usually 48-72 hours post-inoculation), culture fluids were frozen and thawed, clarified by centrifugation at 1000 $\times$ *g* for 15 minutes, and frozen in aliquots at —90°C.

Antisera to prototype adenovirus types 1-18 prepared in horses were kindly made available by the CDC, Atlanta(12). Antisera to types 19-30 were reference reagents prepared in rabbits and distributed by the National Institute of Allergy and Infectious Diseases. Other rabbit antisera (London) against prototype adenoviruses were prepared by injecting rabbits with 2 weekly intramuscular doses of virus in complete Freund's adjuvant, followed a week later by one intraperitoneal dose of

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TABLE I. Adenovirus Strains Recovered from Rectal Swabs, Karachi, 1964.

Number isolated	61
" tested	43
" identified	37
Unidentified	6
Strains against which antiserum was prepared	4

virus without adjuvant. The animals were bled 3 weeks later. Antisera to the Karachi strains were prepared by 4 intramuscular injections of 1.0 ml each of undiluted virus without adjuvant on days 0, 2, 4, and 21. The animals were trial bled at 21 days and exsanguinated on day 28. Dr. Leon Rosen, Pacific Research Section, NIAID, Honolulu, kindly supplied rabbit antisera to adenovirus prototypes 16 and 21 as well as to a type 16 variant designated as the Hunter strain(5). Dr. Rosen also provided the Hunter strain of adenovirus and kindly performed some HI tests with Karachi strains 1 and 2.

Neutralization (Nt) and hemagglutination-inhibition (HI) tests. All Nt tests were performed as previously described(11,13). HI techniques followed Rosen's procedure(1). When isolates were recovered which were not identifiable by HI or Nt, they were purified either by triple plaque passage or by triple terminal dilution passage in human kidney cells. After this purification the isolate was retested with the known antisera. If the strain was still unidentified or exhibited unusual reactions, a virus stock was prepared and antiserum was made in rabbits.

Results. Incidence of unidentifiable strains. Table I indicates the incidence of adenovirus strains which could not be identified by routine HI and Nt screening procedures. Of 43 isolates, 37 could be identified without the necessity of preparing antiserum to the isolate. A wide variety of serotypes was represented: types 1, 2, 3, 4, 5, 6, 7, 8, 10, 16, 18 and 21. These findings were similar to those of a recent study which dealt with isolates from children, where a number of serotypes were present(2).

Karachi-1. The results of the cross HI and Nt tests with the strain designated Karachi-1 are shown in Table II. The type 21 serum distributed by the NIAID did not inhibit Karachi-1 in the HI test although the homologous reaction was low (1:20). The relation of the Karachi strain to prototype 21 was noted by HI only when sera from other sources were used. Sera prepared against Karachi-1 inhibited the hemagglutinin of prototype adenovirus type 21 almost as well as it inhibited Karachi-1. It failed to inhibit 4 units of hemagglutinin of other prototype strains that hemagglutinate rhesus erythrocytes, with the exception of type 14. Type 14 was not tested because of low and variable hemagglutinin titers of this virus, a finding noted by others(14).

The Nt tests yielded results that were somewhat more complicated. Significant titers were obtained with two type 21 antisera when tested against Karachi-1. However, the NIAID serum, which yielded a homologous titer of 1:3840, barely neutralized the virus (serum titer of 1:10). The titers of the other type 21 antisera against Karachi-1 were 4-fold and 64-fold less than in the homologous reac-

TABLE II. Cross Hemagglutination-Inhibition and Neutralization Tests with Karachi-1 and Prototype Adenovirus Types 14 and 21 and Various Antisera.*

Test	Virus strain	Antisera: Adeno 21 (NIAID)	Adeno 21 (London)	Adeno 21 (Rosen)	Karachi-1 (Houston)	Adeno 14 (CDC)
Hemagglutination-inhibition	Adeno 21	20	320	320	80	0
	Karachi-1	0	240	160	120	0
Neutralization	Adeno 21	3840	6400	10,240	120	0
	Karachi-1	10	1600	160	960	80
	Adeno 14	0		0	0	640

* Numbers represent serum titers expressed as the reciprocal of serum dilution. Numbers underlined represent homologous reaction. 0 indicates serum titer of less than 10. A blank indicates test not performed.

TABLE III. Cross Hemagglutination-Inhibition and Neutralization Tests with Karachi-2, Adenovirus 16′ (Hunter) and Prototype Adenovirus Types 14 and 16 Employing Various Antisera.

Test	Virus strain	Antisera: Adeno 16 (CDC)	Adeno 16 (London)	Adeno 16 (Rosen)	Adeno 16′ (Rosen)	Karachi-2 (Houston)	Adeno 14 (CDC)
Hemagglutination-inhibition	Adeno 16	20*	480	80	40	60	0
	Adeno 16′			10	160		0
	Karachi-2	10	240	80	80	160	0
Neutralization	Adeno 16	80	800	1280	160	80	0
	Adeno 16′	10		20	640	1280	20
	Karachi-2	10	400	40	1280	3840	80
	Adeno 14	0				1280	640

* Numbers represent serum titers expressed as the reciprocal of serum dilution. Numbers underlined represent homologous reaction. 0 indicates serum titer of less than 10. A blank indicates test not performed.

tion with London and Rosen's serum, respectively.

Antiserum prepared against Karachi-1 had a homologous neutralization titer of 1:960. An eightfold lower titer was noted with the prototype adenovirus type 21. Therefore, in Nt tests crossing between adenovirus 21 and Karachi-1 was obtained only at low levels.

Another low level cross was found in one direction with type 14 antiserum which inhibited Karachi-1 virus to a titer of 1:80. However, Karachi-1 serum had no neutralizing effect on prototype adenovirus 14 supplied from three different sources.

Karachi-2. Results of cross HI and Nt tests with the strain designated Karachi-2 are shown in Table III. In the HI tests the similarity between adenovirus 16 and Karachi-2 was evident when sera with high homologous HI titers were employed. When the CDC serum, with a low homologous HI titer of 1:20, was used, only a barely detectable titer (1:10) was obtained with Karachi-2. The other two sera prepared against prototype adenovirus 16 have HI titers close to the homologous level. Antiserum to Karachi-2 inhibited prototype adenovirus 16 to the same titer as in the homologous reaction when tested by HI. When tested by serum neutralization, differences between Karachi-2 and prototype adenovirus 16 became apparent. Although one serum against prototype 16 neutralized Karachi-2 only twofold less than the homologous reaction, other adeno 16 sera showed markedly lower titers against Karachi-2. The titers noted for the Karachi-2 antiserum were threefold higher against Karachi-2 than against adenovirus 16′ (Hunter) but were 48-fold higher titer in the homologous reaction compared to the reaction with prototype adenovirus type 16.

Although Karachi-2 was not inhibited by antiserum against prototype adenovirus type 14 when tested by HI, a titer of 1:80 was noted by neutralization. Whereas the reaction between adeno 14 antiserum and Karachi-1 adenovirus was a one way cross (serum against Karachi-1 did not neutralize prototype adenovirus type 14), serum prepared against Karachi-2 inhibited adenovirus type 14 to a titer of 1:1280. Rosen also reported that adeno types 14 and 16′ reacted in a two way cross(5).

Subsequent study of the strains listed in Table I which could not be identified by the reference typing sera indicated that two were antigenically identical to Karachi-2, that is, they were not identified by HI or Nt using CDC sera, but were inhibited by Karachi-2 serum. Another Karachi strain identified as adenovirus 16 was found to be closely related to the prototype 16 and was only slightly related to Karachi-2. The strains that proved difficult to type were tested at both the 3rd and 9th passage levels in both human kidney and KB cells, and were not found to differ significantly in the early and later passages.

Karachi-3. In the course of typing the strain designated Karachi-3, it was clear by

TABLE IV. Cross Hemagglutination-Inhibition and Neutralization Tests with Karachi-3 and Prototype Adenovirus Types 3 and 7 and Various Antisera.

Test	Virus strains	Antisera: Adeno 3 (CDC)	Adeno 7a (CDC)	Adeno 7a (Houston)	Karachi-3 (Houston)
Hemagglutination-inhibition	Adeno 3	60	40	0	160
	Adeno 7	0	320	40	0
	Karachi-3	0	0		160
Neutralization	Adeno 3	1280	0		6400
	Adeno 7	0	640		40
	Karachi-3	1280	80		6400

* Numbers represent serum titers expressed as the reciprocal of serum dilution. Numbers underlined represent homologous reaction. 0 indicates serum titer of less than 10. A blank indicates test not performed.

neutralization that this strain was antigenically related primarily to type 3, but showed crossing with type 7a, as shown in Table IV. Of significance was the fact that no HI activity was detected against this strain in spite of the homologous titers noted with the prototype sera against prototype viruses. Similar strains have been recovered in Houston in the past 4 years (Parks, unpublished data).

When antiserum was prepared against Karachi-3, the titer against the prototype 3 by HI or Nt was equal to the homologous titer. By HI, no reaction occurred with type 7a, but by neutralization a low level crossing was noted in both directions. Of interest was the one way HI cross with type 3 detected using CDC type 7a antiserum. Sera prepared in Houston against prototype adenovirus type 3 and 7a gave no heterologous reactions with each other. However, their homologous HI titers (shown only for type 7a) were lower than those of the CDC sera.

Karachi-4. This strain was found to be a hemagglutinating strain of adenovirus type 25. While the prototype produced very little or no hemagglutinin in our hands, Karachi-4 grown under the same conditions yielded an HA titer of 1:64. Antiserum prepared in rabbits against Karachi-4 yielded serum with HI and Nt titers against both the prototype adenovirus 25 and Karachi-4, although the Nt titers were lower than those noted using the prototype serum.

Discussion. The results from the present study indicated 1) that the majority of adenovirus isolates from Karachi in 1964 could be routinely identified using HI and Nt tests, 2) that with sera of high homologous titers, antigenic grouping of variant strains could be accomplished if one used a *combination* of HI and Nt tests and 3) that strains with significant antigenic differences from the prototype strains are in existence.

One such intratypic variant, Karachi-1, demonstrated the most marked variation when tested by serum neutralization. By HI, Karachi-1 appeared almost identical with prototype 21 when antisera with high HI titers were used. When these same sera were tested by Nt a significant 2-way difference was noted between the prototype and Karachi-1. This pattern closely resembles the pattern observed with adenovirus types 7 and 7a (15). A variant of type 21 adenovirus has not previously been reported, although this type has been a relatively common isolate in some areas(16). Dowdle (personal communication) has recently characterized a type 21 variant which cross reacts with type 16.

Similar patterns of cross reactions were seen with two other variants, Karachi-2 and Karachi-3. Rosen described a variant of type 16 adenovirus, a strain very similar to Karachi-2 which also reacted with adenovirus type 14 antiserum(5). The prevalence of strains similar to these may be fairly high, as witnessed in the present study where 3 of the 4 type 16 strains recovered behaved as variants. The significance of the cross-reaction with the serologically distinct adenovirus type 14 is not known, but may reflect the

genetic relatedness of these two types when tested by other methods such as by DNA guanine-cytosine ratios and by oncogenic potential in newborn hamsters(17).

Whereas the majority of the 45 strains of adenovirus types 3 and 7 isolated in this laboratory in the last 4 years have reacted with one or the other of the prototype sera, but not both, we have recovered 3 strains from different studies at different times which reacted with both type 3 and type 7 antisera by neutralization. One of these strains, Karachi-3, was purified by triple plaque purification, and antiserum was prepared in rabbits. No HI activity was detectable against the strain with prototype 3 or 7a antisera; however Karachi-3 antiserum inhibited prototype adenovirus 3 to homologous HI titer but did not react with type 7a. By neutralization, a low level 2-way cross was noted between adenovirus 7a and Karachi-3; Karachi-3 and adenovirus 3 reacted to equal titers in both directions. It would seem that Karachi-3 is predominantly type 3 with some antigenic relationship with type 7. Other intertypic strains with type 3 and type 7 antigens have been reported; however, they seemed to be even more cross reactive (4,6,7,18). Other adenovirus variants belonging to Group II (rat-positive, rhesus-negative hemagglutinating group) have been described(8,9).

The variants encountered in the present study are obviously of practical importance to those attempting to type adenovirus isolates using only serum neutralization for identification. Unless sera are available with the ability to detect the wide antigenic variation in field isolates of adenovirus, problems of serologic grouping will continue to be encountered. Since such sera are not currently available, it seems best to use HI as a preliminary means of grouping and typing adenovirus isolates as has been recommended by Rosen(5) and as also employed by others(2). However, if the homologous HI titers in the sera used to identify isolates are not high enough (as they obviously were not with the CDC type 3, 7a and 16 typing antisera and the NIAID type 21 antisera), a considerable problem will be encountered in using only HI for routine identification of isolates. Consequently it seems that both HI and Nt are necessary to identify routine isolates.

Even among adenovirus strains already classified as prototypes, marked antigenic overlap exists, especially between types 8 and 9, 10 and 19, 15 and 25, 15 and 22, and the 12-18-31 complex. A similar situation has been noted among enteroviruses, and recently an antigenic grouping was proposed for the group A coxsackievirus type 20 complex whose individual variants had been designated 20, 20A, 20B and 20C(19). Perhaps analogous solutions might eventually be necessary for the human adenoviruses.

Intratypic and intertypic variants present practical problems in identification and consequently serious consideration should be given to the preparation of broad antisera against such strains. Otherwise the number of "new" adenovirus prototypes might continue to increase indefinitely.

Summary. From the 43 adenovirus strains recently isolated from material collected in Pakistan, a number of antigenic variants were recovered. Strain Karachi-1 was found to be closely related to adenovirus type 21 by hemagglutination-inhibition but was significantly different in neutralization tests (Nt). Karachi-3 was antigenically related to adenovirus types 14 and 16. By HI the reaction between type 16 and Karachi-2 was clear, although by Nt significant differences were noted. Karachi-3 was not inhibited in the HI test by either type 3 or type 7a antisera but was found to be predominantly type 3, with a low level two-way cross with type 7a, when tested by Nt. Karachi-4 was found to be a hemagglutinating strain of adenovirus type 25, unlike the prototype in our hands.

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Influence of Bradykinin on Water, Sodium, Potassium, Magnesium and Calcium Content of Segments of Arteries and Veins of Dogs *in vitro*.* (32131)

C. C. HUANG, N. P. DEPASQUALE, AND G. E. BURCH
Department of Medicine, Tulane University School of Medicine and the Charity Hospital of Louisiana, New Orleans

Bradykinin is a powerful vasoactive polypeptide. Its role in inflammation, tissue injury, reactive hyperemia, and the regulation of the circulation has received attention from many laboratories including our own(1,2,3). Because of its strong vasoactivity and because of our interest in its action on postcapillary blood vessels including A-V shunts, it was considered of interest to learn the effects of bradykinin upon electrolyte content of arteries and veins which were available from dogs under study of hemodynamic phenomena. Such information is of importance because of the role of sodium, potassium, magnesium and calcium in smooth muscle metabolism and contraction.

Material and methods. Dogs were anaesthetized with pentobarbital, and their femoral arteries and veins were rapidly dissected and placed in Krebs bicarbonate solution(4), oxygenated by bubbling a mixture of 95% oxygen and 5% carbon dioxide. The temperature of the water bath was maintained at 38.5°C(5). Two beakers, each containing about 50 ml of oxygenated Krebs bicarbonate solution, were also kept in the water bath, and to one was added 1 μg/ml synthetic bradykinin.† Two segments of femoral artery and two segments of vein were transferred simultaneously, one of each into both beakers, and removed 10 minutes later. Any solution adhering to these vessels was blotted away with filter paper. After weighing, the samples were placed in an oven at 105°C for 48 hours and were then reweighed. The samples were digested over an open flame with HNO_3, H_2SO_4 and $HClO_4$, as previously described (6). The sodium, potassium, magnesium, and calcium contents of the vessel segments were determined by flame photometry (Zeiss PMQII, double monochromator)(7).

Results. The water content of the arteries and veins was not significantly changed by bradykinin (Table I). After treatment with bradykinin there were statistically significant changes in the electrolyte content in the arterial wall (Table I). The amount of sodium decreased from 155.5 ± 20.7 mEq/kg

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