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Restriction Endonucleases in Identification of a Genome Type of Adenovirus 19 Associated with Keratoconjunctivitis

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Adenovirus type 19 (Ad19) was first associated with disease in 1973 when several outbreaks of keratoconjunctivitis were reported from Europe and North America. We have examined Ad19 isolates by deoxyribonucleic acid restriction with *Bam*HI, *Bgl*I, and *Sma*I restriction endonucleases. All keratoconjunctivitis-associated Ad19 isolates were identical but different from the Ad19 prototype. The total number of resolved restriction fragments of the Ad19 prototype genome was 31, only 17 of which migrated as the restriction fragments of keratoconjunctivitis-associated genomes. We conclude that two different genome types of Ad19 exist, one of which has been responsible for the recent outbreaks of keratoconjunctivitis.

Bell et al. (1) first isolated adenovirus type 19 (Ad19) to Ad24 in the course of an investigation of trachoma in Saudi Arabia from 1955 to 1956. A high prevalence (85%) of Ad19 infections in Africa was suggested by serological evidence (6). In Europe the prevalence of seropositive individuals has been low; none of 40 Belgians (6) and 15% of the Germans displayed antibodies against Ad19 (15). No reports on the association of Ad19 with outbreaks of disease were reported until recently. Since 1973, Ad19 has been associated with outbreaks of conjunctivitis in Belgium, Canada, Czechoslovakia, Holland, the United Kingdom, and the United States (4-6, 8, 9, 11, 13, 18, 21, 25, 27, 28).

By restriction analysis we have recently identified three different genome types of Ad7 which differ in their degree of pathogenicity. The present report is a similar analysis of six Ad19 isolates. We conclude that a new Ad19 genome type (Ad19a) is associated with epidemic outbreaks of keratoconjunctivitis.

MATERIALS AND METHODS

Virus strains. The prototype strain 587 of Ad19 was obtained from M. Johansson (National Bacteriological Laboratory, Stockholm, Sweden). This strain was plaque purified in A549 cells. Deoxyribonucleic acids (DNAs) from 10 clones were restricted with *Sma*I and found to be identical. Strains 73-20435 and 73-20436 were isolated by J. Desmyter (Rega Institute, Leuven, Belgium) during the fall of 1973 from cases of bilateral keratoconjunctivitis. They were analyzed after passage in GB_2, HL_2, and A-549 cells. Strains 74-5113, 74-5114, and 74-5115 were isolated by K. W. Slaterus (Amsterdam, The Netherlands) during December 1973 and January 1974 from two cases of keratoconjunctivitis and one case of conjunctivitis. The strains were analyzed after passage in GB and A-549 cells. GB and HL are diploid human fibroblast lines. A-549 cells were obtained through the courtesy of W. A. Nelson-Rees. All strains reacted as Ad19 in neutralization tests performed by the method of Rowe et al. (23), in hemagglutination-inhibition tests performed as previously described (29), and in a type-specific immunofluorescence assay performed by the method of Johansson and Wadell (14).

Preparation of viral DNA. The Ad19 strains were propagated as previously described (33). Extraction of intracellular viral DNA was performed by a modification of the method described by Hirt (12). Confluent monolayers of A-549 cells in 275-cm^2 bottles were harvested 2 to 5 days postinfection. Cells were pelleted at 1,500 rpm in a GSA rotor (Sorvall) at 4°C, rinsed twice, and suspended in TE buffer [10 mM tris(hydroxylmethyl)aminomethane (Tris)-hydrochloride, pH 7.0-10 mM ethylenediaminetetraacetate]. Sodium dodecyl sulfate (BDH, Poole, England) was added to a final concentration of 0.6%. The samples were incubated for 10 min at room temperature followed by 5 min at 60°C.

Pronase B (Calbiochem) predigested for 90 min at 37°C was added to a final concentration of 2 mg/ml, and incubation continued for 60 min at 37°C. NaCl (5 M) was added dropwise to a final concentration of 1 M. After gentle mixing for 10 min, the lysate was stored overnight at 4°C. After sedimentation at 17,000 × *g* for 30 min at 4°C, ribonucleases A and T_1 were added to final concentrations of 30 mg/ml and 80 U/ml, respectively, and incubated at 37°C for 120 min. The ribonuclease preparations had been preincubated at 80°C for 30 min. The incubation at 37°C was continued for 4 h after a second addition of pronase B to a final concentration of 1 mg/ml. The DNA was then extracted three times with phenol and three times with ether. DNA was finally precipitated with 2 volumes of ethanol at −20°C, resuspended in 10 mM Tris (pH 7.9), and stored at 4°C after addition of 10 μl of chloroform.

The viral DNA was analyzed by electrophoresis in 0.8 to 1.2% (wt/vol) agarose slab gels as previously

described (26). Ad2 DNA cleaved with *Eco*RI or *Hpa*I and Ad7b DNA cleaved with *Bam*HI were used as molecular weight references. Ad2 DNA is cleaved into six fragments by *Eco*RI (13.45×10^6, 2.80×10^6, 2.369×10^6, 1.725×10^6, 1.45×10^6, and 1.196×10^6) (19) and into seven fragments by *Hpa*I (6.992×10^6, 6.302×10^6, 4.554×10^6, 3.243×10^6, 1.010×10^6, 0.552×10^6, and 0.345×10^6) (19). *Bam*HI cleaved Ad7b into 10 fragments (6.25×10^6, 5.27×10^6, 2.92×10^6, 2.48×10^6, 2.25×10^6, 1.67×10^6, 0.67×10^6, 0.54×10^6, 0.46×10^6, and 0.43×10^6) (31). DNA fragments which are smaller than 1% of the unit length DNA are not resolved by the techniques used.

Restriction endonucleases. Restriction endonuclease *Bam*I was purified by the method of Bickle et al. (3). *Sma*I was kindly provided by Gunilla Isaksson (Lund, Sweden) and *Bgl*I was purchased from New England Biolabs. *Bam*I was incubated in 10 mM $MgCl_2$–10 mM Tris-hydrochloride (pH 7.9)–6 mM 2-mercaptoethanol, *Bgl*I was incubated in 66 mM KCl–10 mM $MgCl_2$–10 mM Tris-hydrochloride (pH 7.4)–1 mM dithiothreitol, and *Sma*I was incubated in 20 mM KCl–6 mM $MgCl_2$–10 mM Tris-hydrochloride (pH 9.2).

All enzyme reactions were carried out for 2 h at 37°C. Reactions were stopped by addition of ethylenediaminetetraacetate to a 5 mM final concentration.

RESULTS

Identification of keratoconjunctivitis-associated adenovirus isolates with serological techniques. The hemagglutinating activity of the keratoconjunctivitis-associated isolates and the Ad19 prototype was inhibited by sera against the prototypes of Ad10 and Ad19. However, they all reacted as the Ad19 prototype in the neutralization test (Table 1). Since the keratoconjunctivitis-associated isolates were indistinguishable from the prototype of Ad19 when analyzed with conventional serological typing procedures, a detailed analysis of their genomes was required.

DNA restriction with *Bam*HI. *Bam*HI recognizes the sequence 5'G GA TCC (22). Ad19 prototype genome was cleaved into fragments A, B, and C ranging in molecular weight from 6.5×10^6 to 8.5×10^6. DNA from the five keratoconjunctivitis-associated Ad19 isolates displayed identical cleavage patterns with *Bam*HI fragments a, b, and c with molecular weights of $>14.0 \times 10^6$, 6.0×10^6, and 1.45×10^6. None of these migrated as the *Bam*HI fragments of the Ad19 prototype DNA (Fig. 1).

DNA cleavage with *Bgl*I. *Bgl*I recognizes the sequence 5' GCCNNNNNGGC (D. Nathans, personal communication). Thirteen *Bgl*I fragments were resolved after electrophoresis of the restricted Ad19 prototype genome. All 13 DNA fragments from the five keratoconjunctivitis-associated Ad19 isolates cleaved by *Bgl*I comigrated. Only nine of these migrated as the *Bgl*I fragments of the Ad19 prototype genome. Several DNA fragments with a molecular weight $<0.30 \times 10^6$ exist but were not resolved by the separation procedure used (Fig. 2 and Table 2).

TABLE 1. *Serological cross-reactions of Ad10 and Ad19 prototypes and Ad19 isolates associated with keratoconjunctivitis*

Adenovirus strain	Neutralization titer with serum against:		Hemagglutination-inhibition titer with serum against:	
	Ad19 prototype	Ad10 prototype	Ad19 prototype	Ad10 prototype
73-20435	64	<25	80	320
73-20436	128	<25	160	640
74-5113	256	<4	80	320
74-5114	≥512	<4	20	160
74-5115	256	<4	40	160
Prototype 19	**128**[a]	<4	**160**	640
Prototype 10	<25	**256**	320	**1,280**

[a] Data in boldface indicate homotypic titers.

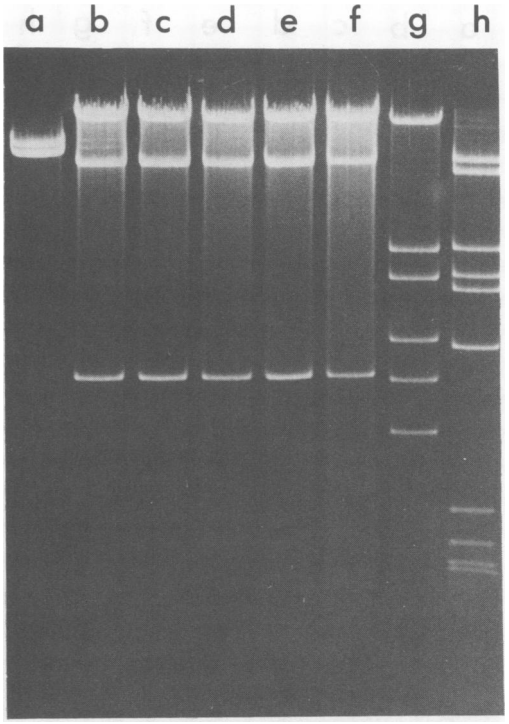

FIG. 1. *Distribution of BamHI DNA restriction fragments after electrophoresis in a 1.2% (wt/vol) agarose gel. Ad19 prototype (a); five keratoconjunctivitis-associated Ad19 isolates (b to f); Ad2 DNA restricted with EcoRI (g); and Ad7b DNA cleaved by BamHI (h). The two faint bands in slot b, which are juxtaposed with the bands in slot a, are spillover from this slot.*

DNA restriction with *Sma*I. *Sma*I recognizes the sequence 5'CCC GGG 3' (7). Fifteen *Sma*I fragments were resolved after electrophoresis of the cleaved Ad19 prototype genome. The five keratoconjunctivitis-associated Ad19 isolates displayed 14 comigrating fragments after digestion with *Sma*I. Eight of these migrated as the *Sma* fragments from the Ad19 prototype genome (Fig. 3 and Table 2).

Thirty-one *Bam*I, *Bgl*I, and *Sma*I fragments of the Ad19 prototype genome were resolved. Only 17 of these migrated as the corresponding fragments of the genomes of the five keratoconjunctivitis-associated Ad19 isolates.

DISCUSSION

The Ad19 prototype was isolated in 1955 from a Saudi Arabian child with trachoma (1, 2). The recent frequently reported outbreaks of Ad19 associated with keratoconjunctivitis, the observation of Ad19 neutralizing antibodies in 2 out of 180 individuals in the United States (25), and the low prevalence of Ad19 neutralizing antibodies in Europe (6, 15) have been interpreted as the arrival of an agent capable of substantial spread (25). This Ad19 strain caused conjunctivitis which was more prolonged with more severe ocularpathology than other contempory cases of viral conjunctivitis (25). Lid edema, preauricular adenopathy, and keratitis occurred among more than two-thirds of the patients from which Ad19 was isolated in 1974 (25). No substantial difference in growth characteristics or physical resistance was noted between the Ad19 prototype strain and the newly emerged Ad19 (21).

The gene products specifying the antigenic determinants responsible for serological identification appear to be shared between the Ad19 prototype strain and the newly appearing Ad19 strains. The suggested increased transmissibility, which characterized the newly appearing Ad19 strains, was expected to be a result of differences in gene products between the Ad19 strains.

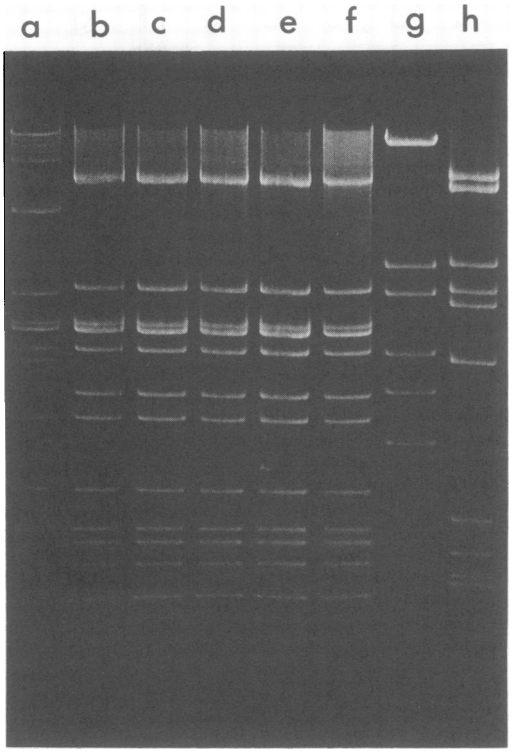

FIG. 2. *Distribution of BglI DNA restriction fragments after electrophoresis in a 1.2% (wt/vol) agarose gel. Ad19 prototype (a), the keratoconjunctivitis-associated Ad19 isolates (b to f), Ad2 DNA restricted with EcoRI (g), and Ad7b DNA cleaved by BamHI (h).*

TABLE 2. *Restriction fragments of Ad19 prototype and Ad19a DNA*

Adenovirus	*Bgl*I fragment (mol wt × 10^{-6})	*Sma*I fragment (mol wt × 10^{-6})
Ad19 prototype	A (4.10)[a]	A (4.50)
	B (2.30)	B (4.35)
	C (1.95)	C (1.95)
	D (1.86)	D (1.85)
	E (1.86)	E (1.72)
	F (1.70)	F (1.46)
	G (1.65)	G (1.28)
	H (1.22)	H (1.24)
	I (1.10)	I (1.11)
	J (0.82)	J (0.58)
	K (0.65)	K (0.58)
	L (0.52)	L (0.53)
	M (0.39)	M (0.53)
	N (<0.30)	N (0.34)
		O (0.32)
Ad19a	a (5.70)	a (6.00)
	b (2.42)	b (3.50)
	c (1.95)	c (3.20)
	d (1.86)	d (1.95)
	e (1.86)	e (1.85)
	f (1.70)	f (1.28)
	g (1.37)	g (1.24)
	h (1.22)	h (0.88)
	i (0.82)	i (0.60)
	j (0.65)	j (0.58)
	k (0.60)	k (0.53)
	l (0.52)	l (0.36)
	m (0.39)	m (0.34)
	n (<0.30)	n (0.32)

[a] Molecular weights were obtained by comparison with Ad2 *Eco*RI, Ad2 *Hpa*I, and Ad7b *Bam*I fragments. Nine *Bgl*I Ad19 prototype and Ad19a fragments comigrated; eight *Sma*I fragments comigrated.

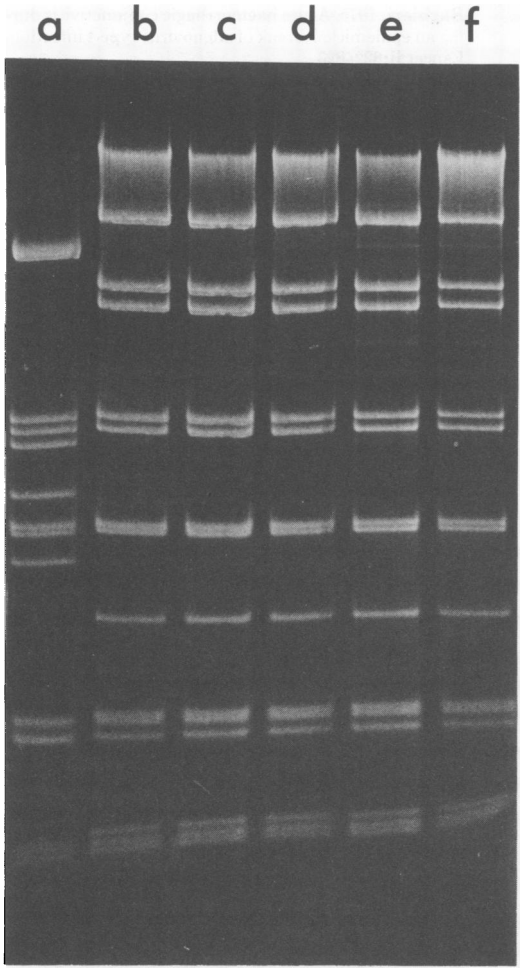

FIG. 3. *Distribution of SmaI DNA restriction fragments after electrophoresis in a 1.2% (wt/vol) agarose gel. Ad19 prototype (a); the keratoconjunctivitis-associated Ad19 isolates (b to f). The discrete DNA fragments, which appear in less than molar amounts with molecular weights >10 × 10^6, are partial digests of the Ad19 prototype DNA (a).*

We have previously demonstrated that three distinct entities of viral genomes, designated genome types, which share the antigenic determinants of the Ad7 prototype can be identified by DNA restriction (31).

Restriction analysis is a most sensitive method for comparison of closely related genomes, second only to nucleotide sequence analysis. The sensitivity can be increased by using several suitable restriction enzymes.

The genomes of Ad19 isolates obtained from five patients with characteristic symptomatology (keratoconjunctivitis) in different countries displayed identical restriction patterns. Thirty-one restriction fragments were resolved after restriction of Ad19 prototype DNA with *Bam*HI, *Bgl*I, and *Sma*I. Only 17 of these could be identified in DNA from keratitis-associated Ad19 strains. We consequently conclude that the newly appearing Ad19 represents a genome type (Ad19a) distinct from the Ad19 prototype. It should be noted that the discordance between the two Ad19 genome types is larger than was noted in the analysis of Ad7 (31).

Genetic variation during in vitro passage of adenovirus is apparently limited, since 10 clones of the Ad19 prototype displayed identical restriction patterns. Furthermore, the Ad7a original isolate and the Ad7 vaccine strains showed the same DNA restriction patterns upon analysis after 17 to 22 passages in vitro as wild-type isolates analyzed after the second passage in vitro (31).

It has been suggested that Ad8, Ad9, Ad10, and Ad19 should be treated as a subgroup associated with epidemic conjunctivitis (21). This pathogenetic property is a poor criterion for classification. The 35 established adenovirus types have been divided into five subgroups (10, 30), many of which harbor members which are associated with eye infections. In subgroup B, Ad3, Ad7, Ad14, and Ad16 have been isolated from cases of follicular conjunctivitis (24) and Ad11 has been associated with keratoconjunctivitis (17). In addition to the four serotypes from subgroup D suggested to form a new subgroup (21), Ad15, Ad17, Ad20, and Ad29, which are members of subgroup D, also have been associated with eye infections (16, 24). Finally Ad4 (subgroup E) has been associated with outbreaks of keratoconjunctivitis (8, 20).

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