Identification of the Kirk "Hepatitis" Virus as a Member of the Parvovirus (Picodnavirus) Group (35955)

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The Kirk (K) virus was independently recovered in two laboratories from a cloned line of Detroit-6 (D6) cells inoculated with plasma of a volunteer taken 30 and 37 days after ingestion of MS-1 infectious hepatitis serum (1, 2). The properties of the agent (2) were similar to those of the picodna- or parvoviruses (3), and led to the studies reported below on the identification of the K virus.

Materials and Methods. Tissue culture systems. A cloned line (No. 82) of D6 cells in its seventh passage was obtained from J. D. Boggs and E. Caldwell. For this study, cells were used from the 10th to the 19th passage. Twice during the study the cells were tested for presence of mycoplasma, with negative results. Propagation and maintenance techniques were identical to those reported for other D6 lines (1, 2). Rat embryo (RE) and hamster embryo (HE) tissue cultures were prepared from embryos in midgestation. Only low passages (fourth–sixth) of RE and HE cells were employed for virus assays. Secondary cultures of human embryonic kidney (HEK) cells and primary cultures of green monkey kidney (GMK) cells were also used (4). Serially propagated cultures of human and simian bone marrow cells were prepared as previously described (5).

Viruses. Kirk (K) virus preparations in D6 cells have been reported (1, 2). The following parvoviruses were used: X14 virus passed in RE cells (6); H-3 virus and Kilham rat virus (RV), kindly supplied by Dr. H. W. Toolan as liver extracts of 5-day-old sick hamsters (7) which had been inoculated at birth. Satellite-free human adenovirus 7a was used in enhancement tests (8).

Immune sera. Two antiserum pools were prepared from hamsters inoculated at birth with K virus and bled 5 weeks later. Pool 1 was from hamsters inoculated with undiluted virus ($10^{3.5}$ TCD_{50}/ml) and pool 2 from animals inoculated with 100 times diluted virus ($10^{1.5}$ TCD_{50}/ml). No illness was observed and the liver, spleen, and kidneys were normal histologically. Rabbit antiserum was kindly provided by Dr. L. R. Overby.

The following series of hamster immune and normal sera (prepared in young adult hamsters) was generously provided by Dr. H. W. Toolan: H-1 (without cross-reactions to H-3, RV, or HB parvovirus); H-3 (cross-reacting with RV); HB (without cross-reactions to H-1, H-3, or RV) and normal hamster serum (without cross-reactions to any of the above viruses). The antisera to the 4 types of adeno-satellite viruses have been described (9).

Virus assays and neutralization (Nt) tests. Inocula were added to drained cultures and allowed to adsorb for 60 min at 37°. Upon addition of maintenance medium, D6 tube cultures were rotated at 34–35° for 5 days, while other cell cultures were kept stationary at 37° for 10–14 days. For the cultures of D6 cells, virus titers were calculated from the readings on day 5 after inoculation, while for the cultures of RE cell this was done on day 10 of incubation. The Nt tests were performed by the constant virus-varying serum dilution procedure. Virus–serum mixtures were incubated for 2 hr at 37° prior to inoculation.

Hemagglutination (HA) and hemagglutination-inhibition (HI) tests. Tests were run in Linbro plastic plates, using a micromodification (total volume 0.2 ml) of the method described by Moore (10). Tests were done

with 0.4% suspensions of guinea pig, rhesus monkey, and human (group O) red blood cells (RBC). The HA titer was expressed as the highest virus dilution causing 100% agglutination, while an HI end point indicated the highest serum dilution which completely inhibited HA of the test cells. All sera used in HI tests were treated with kaolin prior to use, as described (10).

Enhancement studies. Adenovirus 7a was used as helper virus in potentiating K virus replication in D6 and HEK cells. The procedures were similar to those described previously for adeno-satellite viruses (8).

Determination of physical properties and nucleic acid type. Determinations of size and ether sensitivity were performed as already reported (2). Resistance to heat was determined by comparing infectivity of virus preparations after heating for 1, 2, and 4 hr at 56°. The pH stability test was conducted following the method employed in characterization of rhinoviruses (11). Nucleic acid typing was done by comparing inhibition of the virus by 5-bromodeoxyuridine (BUDR) and guanidine hydrocholoride, incorporated into maintenance medium at a concentration of 15 and 50 μg/ml, respectively. Poliovirus (type 1, strain LSc) and herpesvirus (type 1, strain KOS) were included as RNA and DNA virus controls.

Results. Growth of K virus in human and simian tissue culture cells. As already reported (1, 2), K virus can be propagated in D6 cells, attaining relatively low titers (an av of $10^{4.0}$ TCD_{50}/ml). As shown in Table I, no multiplication occurred in any other cell culture tested. This was indicated by an absence of cytopathic changes during a 30 day incubation period.

The sequence of morphological changes induced in D6 cells strikingly resembled those described in detail by Moore (10) for some of the H viruses in the RE cell system. Initial changes (2–3 days after inoculation of the virus) consisted of pronounced granularity in slightly enlarged cells. After 1–2 more days, the affected cells pulled apart, causing a rarefaction of the monolayer. Final changes (days 5–6 of incubation) were characterized by disruption of infected cells with resulting necrotic cytoplasmic debris attached to pyknotic nuclei.

TABLE I. Susceptibility of Various Human and Simian Cell Cultures to K Virus.

Cell system	Virus growth
Detroit 6 (Clone 82)	+ ($10^{4.0}$ TCD_{50}/ml)[a]
Human embryonic bone marrow	—[b]
Chimpanzee bone marrow	—
Baboon fetal bone marrow	—
Marmoset bone marrow	—
Human embryonic kidney	—
Green monkey kidney	—

[a] Average titer on day 5 after inoculation.

[b] Minus indicates no cytopathic changes during 30 days of incubation.

General properties of K virus. Determination of virus size by ultrafiltration confirmed the previously reported observation that the virus is of small size (2). The agent was able to pass through Millipore filters with an average pore diameter of 50 nm, or larger, without any loss in infectivity; it was retained, however, by 25 nm Wright-Flemming gradacol membranes. Since virus the size of polio passes a 50 nm pore but with some loss in titer, the size of K virus was estimated to be slightly smaller than poliovirus, which is 28 nm. This is in agreement with electron microscopic observations of spherical particles with a diameter of 18–20 nm (2). Crude virus preparations contained particles but at a low concentration (less than 10^7/ml); many of the particles were empty (2).

Overnight treatment with 20% ether at 4° did not affect the virus titer, confirming the earlier report (2). Other characteristics of the virus are summarized in Table II. The virus was resistant to low pH and to heating for 2 hr at 56°. However, 90% of the virus was inactivated after heating for 4 hr. Results of tests with chemical inhibitors indicated that K virus contains a DNA genome; guanidine hydrochloride did not suppress virus multiplication, whereas BUDR almost completely (99%) inhibited replication of the virus.

Neutralization tests. A summary of the results is presented in Table III. The K antisera (of both rabbit and hamster origin)

TABLE II. pH Stability, Thermoresistance, and Nucleic Acid Type.
Results are shown as $\log_{10}$ TCD_{50}/ml on day 4 after inoculation of assay cultures.

Virus	pH stability: infectivity after exposure (3 hr at room temp)		Thermoresistance: infectivity after heating at 56° for (hr):				Nucleic acid typing: infectivity in presence of inhibitor		
	pH 2.6	pH 7.3	0	1	2	4	None	BUDR	Guanidine
K	3.5	3.5	3.5	3.5	3.5	2.5	3.5	1.5	3.5
Poliovirus type 1 (LSc)	—			—			7.5	—	2.0
Herpes simplex virus type 1 (KOS)	—			—			4.5	<1.0	—

inhibited the homologous K virus. The virus was also completely neutralized by a high dilution (1:1280) of H-3 antiserum. Antisera to other H viruses (H-1, HB), as well as adeno-satellite antisera, did not exhibit any neutralizing effect in the lowest dilution tested (1:10). The H-3 antiserum neutralized the homologous H-3 virus, yielding a serum titer of 1:10,240. The K antisera inhibited the H-3 and the homologous K virus to about equal titers (1:640–2560). In the same test, sera collected at various stages of K's infection (24 days before to 121 days after ingestion of the MS-1 serum pool) had no detectable antibodies to K virus at dilutions of 1:10. The same negative findings were made for sera of 3 laboratory workers (after they had worked with the virus for 6 months) and for 4 different preparations of human gamma globulin diluted 1:10.

TABLE III. Results of Neutralization Tests.

Antisera	50% serum titer in presence of: K virus[a] (32 TCD_{50})	H-3 virus[b] (100 TCD_{50})
K antisera		
Rabbit	2560[c]	2560
Hamster pool 1	1280	1600
Hamster pool 2	1280	640
Parvovirus antisera		
H-1	<10	
HB	<10	
H-3	1280	10,240
Adeno-satellite type 1	<10	
2	<10	
3	<10	
4	<10	

[a] Conducted in D6 cells.
[b] Conducted in RE cells.
[c] Reciprocals of serum dilution.

HA tests. Except for adeno-satellite types 1–3, the parvoviruses are known to agglutinate RBC of different species. Consequently, K virus was tested for that capacity using human (group O), rhesus monkey, and guinea pig RBC. The HA patterns of K and viruses of the H-3 complex (antigenically related H-3, RV, and X14) are shown in Table IV. All the viruses agglutinated guinea pig RBC, with titers ranging from 1:640 (K virus) to 1:5120 (H-3 virus). K and RV were the only viruses that agglutinated human RBC. X14 virus agglutinated rhesus monkey cells to a dilution of 1:640, while RV and H-3 had the same capacity but in much lower titers (1:40 and 1:10, respectively). K virus failed to agglutinate rhesus monkey RBC in the lowest dilution tested (1:10).

TABLE IV. Results of Hemagglutination Tests.

Virus (source; cell passage)[a]	HA titers[b] vs indicated RBC: Human O	Monkey	Guinea pig
K (D6; 5)	40	<10	640
X14 (RE; 2)	<10	640	1280
H-3 (liver filtrate)	<10	10	5120
RV (liver filtrate)	320	40	2560

[a] Control cell cultures (D6, RE) were negative in 1:10 dilution.
[b] Reciprocals of virus dilution.

TABLE V. Results of Cross Hemagglutination-Inhibition Tests.

Virus (source; cell passage)	HI serum titers[a]				HA units present in test (guinea pig RBC)
	Anti-H-3 hamster	Anti-Kirk			
		Hamster			
		pool 1	pool 2	Rabbit	
K (D6; 5)	800	3200	800	1600	4
X14 (RE; 2)	800	640		400	4
H-3 (liver filtrate)	3200	1600	400	1600	4
RV (liver filtrate)	800	640		400	4

[a] Reciprocals of serum dilution.

Cross HI tests with K, H-3, RV, and X14 viruses. As demonstrated above in the neutralization tests, K virus was neutralized by its own antiserum and antiserum to the H-3 virus. To determine the degree of HI cross-reactivity between the two viruses, tests were conducted as illustrated in Table V. The H-3 antiserum had the highest HI titer with the homologous H-3 virus (1:3200), but the serum cross reacted to high titers (1:800) with the other test viruses. K antisera reacted to equally high titers with both the homologous K virus and the H-3 virus (1:1600–3200). The same sera crossed with RV and X14 viruses but yielded 4- to 5-fold lower titers.

Growth pattern of K and other H-3 related viruses in D6, RE, and HE cells. Two criteria were used to evaluate the multiplication of the respective virus in a particular cell culture: (a) the appearance of cytopathic effect (CPE) during incubation of 5 days (in D6 cells) to 14 days (in RE and HE cells); and (b) detection of HA activity for guinea pig RBC at the end of the incubation period. As shown in Table VI, in D6 cells, K and H-3 virus induced cytopathic changes in parallel with the HA activity, while RV and X14 viruses multiplied without causing CPE. In RE cells, K virus was unique among the H-3 related viruses. It failed to produce cytopathic changes or hemagglutinin, even after 3 passages, whereas the other members of the H-3 group grew best in the RE cells. None of the viruses induced CPE in HE cells. Nevertheless, they all replicated to a certain extent, as evidenced by detection of various degrees of HA activity.

TABLE VI. Susceptibility of D6, RE, and HE Cells to K and Other Viruses of the H-3 Group.

Virus	Cells		
	D6	RE	HE
K	+[a] (1:640)[b]	— (<1:2)	— (1:8)
X14	— (1:8)	+ (1:1280)	— (1:16)
H-3	+ (1:128)	+ (≧1:4096)	— (1:512)
RV	— (1:16)	+ (1:256)	— (1:64)

[a] + or — indicates presence or absence of CPE.

[b] HA titer of the last tissue culture passage is given in parentheses. Viruses were passaged at least twice in the respective cells. Fluids from all control cell cultures were negative for HA at 1:2 dilution.

Adenovirus as helper. In the course of these studies, it was established that K virus did not replicate in HEK cells whether inoculated alone, or with adenoviruses. However, enhancement of growth by adenovirus 7a occurred in D6 cell cultures. Results of an illustrative titration are presented in Table VII. Harvests of D6 cell cultures inoculated with tenfold dilutions of K virus alone, or together with adenovirus helper, were first heated for 1 hr at 56°. This temperature inactivates adenovirus but not K virus. Harvests of the virus dilutions were then each titrated for HA activity using guinea pig RBC. As shown, adenovirus 7a enhanced the replication of K virus, the titers being 100 times higher in preparations of cells co-infected with the helper.

Discussion. The K virus recovered from the acute phase plasma of an MS-1 infectious hepatitis patient was found to possess the properties of a parvo- or picodnavirus. The

TABLE VII. Enhancement of K Virus by Helper Adenovirus 7a.

Inoculum		HA titers of dilutions (reciprocals) of virus harvests					
K virus	Adenovirus	$10^{-1.0}$	$10^{-2.0}$	$10^{-3.0}$	$10^{-4.0}$	$10^{-5.0}$	$10^{-6.0}$
+	+	≥1280	≥1280	1280	160	20	<10
+	—	≥1280	1280	20	<10	<10	<10
—	+	<10	—	—	—	—	—

virus is small (18–20 nm in diameter) and has a DNA genome. It is resistant to heating at 56° for 2 hr, to pH 2.6, and to ether. It agglutinates guinea pig RBC, as do certain other parvoviruses.

The K virus was not inhibited by immune sera against 6 different parvoviruses (H-1, HB, and the four adeno-satellite types). However, it was neutralized by three different lots of homologous antisera and also by H-3 antiserum. To further delineate this relationship, K virus was compared in cross-HI tests with the viruses of the H-3 group. These results confirmed the cross-reactivity between the H-3 antiserum and the K virus. Antisera to K virus prepared in hamsters or rabbit crossreacted with all members of the H-3 group, although the serum titers were highest against the H-3 virus. These data imply a strong antigenic relationship between H-3 and K virus.

With the exception of the adeno-satellite viruses, the other members of the parvovirus group are considered capable of autonomous replication. However, at least one of them (H-1) is conditionally defective, being able to replicate in human embryonic lung cells only in the presence of human adenovirus type 12 (12). Similarly, human adenovirus 7a was found to act as a helper for K virus in D6 cells. In repeated tests HA titers of K virus were found to be up to 100 times higher in co-infected cells than in cultures without the added helper adenovirus. A 4-fold increase in the HA titer of X14 virus was also achieved in RE cells in the presence of the same helper virus.

The growth pattern in RE cells revealed the clearest difference between K and other viruses of the H-3 group, as K virus did not replicate in RE cells in the course of three consecutive passages.

The origin of K virus deserves comment. In an earlier study, using cells from a different D6 clone, neutralizing antibodies to the K virus in its earlier passages were found in patient K's convalescent serum. This could not be repeated with the late passages of K virus in the new D6 cloned line. It may be that K virus originated in another species and is not infectious for man. The present findings do not support an etiological role of K virus in infectious hepatitis. If the virus occurred in the patient's blood, it may have been there as a passenger along with the hepatitis virus. A more plausible explanation is that the D6 cells contain K virus as a latent agent which was activated by the addition of the hepatitis sera.

Other parvoviruses may exist in cells as inapparent genomes (13). In the presence of one kind of helper virus, infectious parvovirus nucleic acid may be detected in the absence of virion assembly (14), and in the presence of a second kind of helper virus the assembled virus is quickly produced. Undoubtedly, there is more to learn about these defective viruses and their activation.

Similar findings on the crossing of the K virus with members of the H-3 group and/or the enhancement of the virus by adenovirus helper, have been made and reported to the authors in recent months by L. Overby, J. Boggs, F. Deinhardt, J. Maynard, and their colleagues. Furthermore, Overby and Boggs studied viruses recovered in D6 cells from volunteers developing hepatitis after administration of Kirk's acute phase plasma. In all instances the viruses were neutralized by the K rabbit antiserum which we found to cross-react with the H-3 group of viruses.

Summary. Kirk virus recovered from D6 cells inoculated with acute phase plasma samples of an MS-1 infectious hepatitis patient

was found to possess the properties of a parvo- or picodnavirus. Antigenically it belongs to the H-3 group, although it has a markedly different host range and a somewhat different hemagglutination pattern. Adenovirus type 7a proved to function as an efficient helper for Kirk virus, producing an increase in virus yields of up to 100-fold.

This work was supported in part by Research Contract DADA 17-67C-7004 from the U.S. Army Medical Research and Development Command, and by Training Grant 5 T1 AI-74 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Mrs. Ruth A. Dixon provided able technical assistance. The generous supply of D6 cells and sera by Dr. J. D. Boggs and his associates (Chicago), and of viruses and antisera by Dr. H. W. Toolan (Bennington, Vermont) and Dr. L. R. Overby (North Chicago) is gratefully acknowledged.

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Received June 3, 1971. P.S.E.B.M., 1971, Vol. 138.