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Rat Leukemia Derived 9H Virus (9HV) I. Properties of the Virus and Evidence for the Development of Heterogeneity in Cell Culture*† (33886)

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(Introduced by M. Michael Sigel)

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A recent communication from this laboratory described the isolation of a filterable hemagglutinating agent, negative for *Mycoplasma*, from leukemias produced in rats by cell-free extracts prepared from chemically induced rat mammary carcinomas (1). A serological relationship between this virus, designated "9H" and rat virus (RV) (2) was reported (1). In a more recent study (3) it was shown that inoculation of the 9H virus (9HV) into newborn rats resulted in peliosis hepatis, a condition characterized by hemorrhagic cysts in the liver. The development of similar lesions in the livers of rats inoculated with certain strains of RV was also observed in another laboratory (4). The present paper reports on the properties of 9HV propagated in the REL line of rat embryo cells (1) since its original isolation from leukemic tissues which in certain aspects differ from those hitherto reported for RV.

Materials and Methods. Chemicals, media, and cell cultures. Cesium chloride was obtained from the Matheson, Coleman and Bell Co., Norwood, Ohio. Bio-Glas-500, a powdered glass material of controlled pore size was purchased from Bio-Rad Laboratories, Richmond, California. The origin of the REL cell line and the maintenance medium (MM) for these cells have been described previously (1). Secondary rat embryo (SRE) cells were propagated in MM enriched with 15% bovine amniotic fluid. The cells were grown as monolayers in culture tubes for use in experiments and in culture bottles for further passage and for the preparation of virus stocks.

Virus. Stocks of 9HV were produced by infecting monolayers of either REL or SRE cells. Cytopathic effects (CPE), manifested by granulation, rounding, and vacuolation of the cells, were evident after 12–14 days of incubation at 37°. The cells and culture fluids were harvested and, following a tenfold concentration at 105,000*g* for 1 hr, the virus was placed in ampoules and stored at —70° until needed.

Virus titrations were done in REL cell cultures according to the procedure described by Dougherty (5). The hemagglutinating (HA)

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titer of the virus was assayed by means of guinea pig erythrocytes as previously described (1), and recorded as the reciprocal of the highest dilution that completely agglutinated the cells.

Density gradient centrifugation. The procedure employed was essentially that described by Breese *et al.* (6). Fractions (15 drops each) were collected after piercing the bottom of the tubes with a Buchler piercing unit. Densities of the samples were determined by comparing the index of refraction measurements, made in an Abbe refractometer, with refractive readings on a precalibrated standard curve obtained with CsCl solutions of known density. The position of the virus was located by performing HA assays on each of the fractions. The HA-positive fractions of each zone were pooled, and the CsCl removed by sedimenting the virus at 105,000*g*, and the pellets resuspended in Hanks' Balanced Salt Solution (HBSS) containing 1% newborn calf serum (NCS).

Physicochemical procedures. McIlvaine's citrate–phosphate buffer, and Sorensen's glycine–NaOH buffer were used for the acid and alkaline pH ranges, respectively. Equal parts of virus and buffer were mixed and incubated in a water bath at 25° for 30 min. An amount of HBSS containing 1% NCS was added to give a virus dilution equal to one-tenth the original. An aliquot from each sample was removed for an HA assay, and the remainder was inoculated into REL cell cultures.

Reagent grade ether and chloroform, respectively, were used for the lipid solvent sensitivity studies. One part of the virus preparation was added to 3 parts of solvent, the tubes were tightly stoppered, and kept in the refrigerator for 4–5 hr with intermittent shaking. Following centrifugation at 1,000*g*, the aqueous phase was withdrawn, and residual solvent removed by bubbling air through it.

In temperature sensitivity experiments, 1–1.5-ml aliquots of the virus preparation in sealed ampoules were completely submerged in water baths at the temperature of observation for 30 min. Following the treatment, aliquots of virus were inoculated into REL cell cultures.

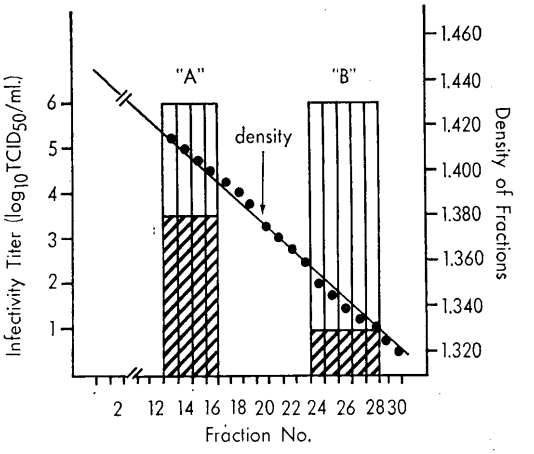

FIG. 1. Cesium chloride sedimentation of the 9HV: the virus was subjected to CsCl density centrifugation in a field-formed gradient of 3.25 *m* CsCl in 0.5 *M* Tris–HCl buffer, pH 7.5. Centrifugation was for 18 hr at 30,000 rpm in the SW 39 rotor, at 4°. The shaded areas represent the infectivity titer of the "A" and "B" components, respectively.

In all experiments the cultures were refed with fresh medium on day 7, and HA determinations were carried out on the harvested culture supernatants 13–14 days postinfection.

Bio-Glas-500 filtration. Columns of porous glass, as reported by Haller (7, 8) were used. One to 2 ml of virus suspension, placed on the columns, was eluted with McIlvaine's citrate–phosphate buffer at pH 7.2. Fractions, 1.5 ml each, of the eluate were collected. The position of the virus was located by performing HA assays on each of the fractions. The HA-positive fractions were pooled and treated as described earlier.

Results. Sedimentation characteristics. Ultracentrifugation of the 9HV in a field-formed CsCl gradient resulted in the appearance of two HA-positive zones as shown in Fig. 1; a heavier one having a mean density of 1.41 and a lighter one exhibiting a mean density of 1.34. These two zones have been termed the "A" and "B" components of the 9HV, or the 9HV-A and the 9HV-B, respectively. Virus from both zones proved to be infectious and cytopathogenic when inoculated into REL cell cultures; this is illustrated in the shaded areas of Fig. 1.

TABLE I. Effect of Temperature on the 9HV and Its Components as Measured by the Production of Progeny Virus in REL Cell Cultures.

Temp (°) of treatment	Hemagglutinating titer:					
	9HV		9HV-A		9HV-B	
	Parental	Progeny[a]	Parental	Progeny[a]	Parental	Progeny[a]
25	16	512	8	128	8	256
56	16	512	8	128	8	128
75	8	512	4	32	4	256
80	2	32	0	0	4	64
86	0	0	0	0	0	0

[a] Day 13 postinfection.

Effect of temperature. The results of experiments to determine the temperature sensitivities of the 9HV and of the individual components are presented in Table I. The heavier "A" component was completely inactivated at 80°, whereas the lighter "B" component required a temperature of 86° to achieve total loss of infectivity. Concomittant treatment of the 9HV complex resulted in the appearance of hemagglutinins and CPE in cell cultures infected with virus treated at 80°

Effect of pH and lipid solvents. Table II shows that the 9HV and its two components are stable to a wide range of pH conditions. However, the 9HV-A which was exposed to a pH of 12 showed both a loss of parental HA titer and the ability to produce hemagglutinating progeny in the host. It thus appears that the 9HV-A exhibits some sensitivity to highly alkaline pH whereas 9HV-B does not since no microscopically detectable nonspecific damage to the host cells was observed following inoculation.

Table II also shows the results of experiments aimed to determine the sensitivities of the 9HV and the components towards two lipid solvents, ether and chloroform. As shown, all three viral preparations retained the ability to produce hemagglutinating progeny following treatment, indicating that if lipid is present in the outer coat of either component it is not essential to the infective property.

Bio-Glas-500 filterability of 9HV-A. The studies described above were carried out with the first and second REL passage of each of the two components. It was observed later

TABLE II. Resistance to pH and Lipid Solvent Treatment by the 9HV and Its Components as Measured by the Production of Progeny Virus in REL Cell Cultures.

Type of treatment	Hemagglutinating titer of:					
	9HV		9HV-A		9HV-B	
	Parental	Progeny[a]	Parental	Progeny[a]	Parental	Progeny[a]
pH						
2.2	16	256	8	128	8	256
5.0	16	128	8	128	8	256
7.4	16	256	8	128	8	256
9.4	16	256	8	64	8	128
12.0	16	256	0	2	8	128
Ether	16	64	8	256	8	128
Chloroform	8	128	8	512	8	256
None	8	64	8	256	8	128

[a] Day 13 postinfection.

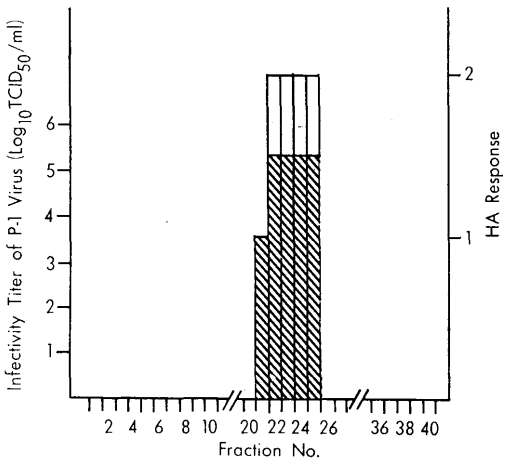

FIG. 2. Bio-Glas-500 column chromatography of 9HV-A: the column was eluted with McIlvaine's citrate–phosphate buffer, pH 7.2, at a flow rate of 1 ml/min. Fractions were collected every 1.5 min. The HA-positive fractions were pooled and treated as described in the text. The "HA response" of the fractions is arbitrarily assigned the nos. 1 and 2, and represent plus–minus and definitive plus, HA patterns, respectively. The superimposed shaded area shows the infectivity titer of the viral progeny obtained after the first (P-1) REL cell culture passage of the filtrate.

that a cesium chloride density centrifugation of the third REL passage of the 9HV-A, which was expected to yield only the heavy 9HV-A component, also revealed the presence of the lighter 9HV-B component. High titered stocks were produced by passing the virus from the heavy zone twice in SRE cell cultures, and harvesting the second passage progeny. Aliquots of this preparation were filtered through Bio-Glas-500 columns. The choice of this material, which has an approximate particle exclusion limit of 50 mμ, was based on the finding, discussed below, that the 9HV-B passes through Gradocol filters of 30 mμ.

Figure 2 shows the results of a representative Bio-Glas-500 filtration experiment. Four such experiments were carried out, and the results obtained were in general agreement. A single HA-positive region of 5 fractions was observed. These were pooled, and exhibited a cumulative HA titer of 16, representing an approximate 1000-fold decrease when compared to the prefiltered material which exhibited an HA titer of 16,384.

Temperature sensitivity of the Bio-Glas-500 filtrate and its progeny. In attempting to ascertain the homogeneity of the viral preparations, temperature sensitivity experiments were carried out on prefiltered 9HV-A, Bio-Glas-500 filtrate directly, and on the progeny of the filtrate, serially passed in REL cell cultures. The results of these experiments are summarized in Table III. Prefiltration parental 9HV-A treated at 80° gave rise to high titered progeny when inoculated into REL cultures, thus indicating the initial presence of appreciable amounts of the 80°-resistant 9HV-B. Aliquots of Bio-Glas-500 filtrate, treated at the temperatures indicated, were each inoculated into 10 separate REL cultures. Progeny were harvested on day 14 postinfection, and following clarifica-

TABLE III. Temperature Sensitivity of 9HV-A (i) Prior to Bio-Glas-500 Filtration; (ii) of the Filtrate, and (iii) the Progeny of the Filtered Virus.

Virus preparation	Temperature of treatment		
	25°	80°	86°
	HA titer of progeny[a]		
Prefiltration 9HV-A:	1024	2048	0
Bio-Glas-500[b] filtrate of 9HV-A P-1			
culture no. 1	4	0	0
2	8	0	0
3	128	0	0
4	64	0	0
5	64	0	0
6	128	0	0
7	4	0	0
8	256	0	0
9	8	0	0
10	128	0	0
Progeny of Bio-Glas-500 filtrate			
P-2[c]	2048	0	0
P-3	64	8	0
P-4	1024	32	0
P-5	32	1024	0

[a] Culture supernatants day 14 postinfection.

[b] HA determinations done on each individual culture.

[c] Number of REL passages undergone by the filtrate.

tion of the culture fluids, HA tests were performed. It was found that whereas appreciable HA-positive material and CPE appeared in the cultures inoculated with filtrate treated at 25°, no hemagglutinins or CPE were detectable in the cultures which were inoculated with filtrate treated at 80 or 86°, indicating the absence of the 9HV-B. Successive REL passages of the Bio-Glas-500 filtered virus resulted in the appearance of progressively increasing HA-positive material when the parental virus was treated at 80°. This would seem to suggest that the growth of the "A" component in REL cell cultures initiates or stimulates the production of the "B" component of the 9H virus.

In order to determine whether the Bio-Glas-500 filtrate was pure 9HV-A and free of the 9HV-B, the 80°-treated filtrate was consecutively passed "blindly" in REL cell cultures. Five such passages failed to reveal the presence of hemagglutinins or CPE in the inoculated cultures. It seems therefore that the Bio-Glas-500 filtration removes the lighter 9HV-B which once again makes its appearance in REL cell cultures as the heavier 9HV-A is passed successively.

Discussion. Evidence that the 9HV, isolated from tissues of leukemic rats and serially passed in REL cell cultures, contains two similar but distinct components, is based on the following observations: (i) the appearance of two infectious hemagglutinating zones following sedimentation in a CsCl density gradient, a finding similar to that reported by Payne *et al.* (9) for the X-14 virus and by Green and Karasaki (10) for the H-1 virus, but dissimilar from the results obtained by Breese *et al.* (3) with Kilham's rat virus in a potassium tartrate density gradient; and (ii) a differential in temperature sensitivity of the two components. In addition to the above, 9HV was filtered through Gradocol membranes of a pore size of 30 mμ. The filtrate was inoculated into REL cell cultures and proved to be infectious as evidenced by the appearance of high titered hemagglutinating virus. The characteristics of the progeny of the filtrate were compared to the 9HV components obtained from CsCl density gradient centrifugation. No evidence was found for the presence of 9HV-A in the filtered virus preparations which proved to be identical in their physicochemical properties to the 9HV-B obtained by density centrifugation.

The serological relationship of the 9HV to Kilham's RV has been reported earlier (1) and was mentioned at the outset. Consequently, RV strain 13 (obtained through the courtesy of Dr. H. J. Spencer) was subjected to CsCl density centrifugation after several serial passages in REL cultures. Only one hemagglutinating zone appeared in the region corresponding to a buoyant density of 1.33, similar to that observed with the 9HV-B, indicating that RV-13 does not contain the "A" component of the 9HV but is similar, or identical to, the "B" component. It would thus appear that the 9HV-B is a new strain of RV closely associated with another component, the 9HV-A.

The present study also revealed that, although Bio-Glas-500 filtration was effective in removing the lighter "B" component from the mixture, the growth in tissue culture of the pure 9HV-A gave rise to the 80°-resistant 9HV-B. Similar observations with other viral systems have been made by several investigators. Hosaka and Kitano (11) reported the fractionation of Sendai virus by sucrose gradient centrifugation into four classes of morphologically distinct infectious virions. However, the progeny produced by each fraction had a heterogeneous distribution similar to that of the original virus. Aurelian and Wagner (12) found that infection of HEp-2 cells with the MP strain of herpes simplex virus gave rise to two populations of virions which differed from each other in heat lability and buoyant density. In the instances cited a difference in infectivity was noted between the members of the progeny virus, whereas the infective property of the 9HV components has so far proven to be similar in its stability when stored at −70° and in virulence; the latter being ascertained by the rapidity in the appearance of CPE, HA, and infectivity titers.

The possibility that the REL cell line carries the 9HV-B latently, and is potentiated

by the infection with the 9HV-A, is unlikely because during the period of this study the uninoculated cultures have been tested for the presence of virus, and the results proved to be negative, in agreement with previous findings (1). It would appear, therefore, that the production, in culture, of the "B" component may result from the "A" component carrying this information in its genome. Attempts to determine the nucleic acid type of RV and related agents have been reported. Jamison and Mayor (13) used acridine orange to stain purified rat virus, strain X-14, and postulated, on the basis of their results, that X-14 might be a single-stranded virus. Cheong *et al.* (14), however, on the basis of preliminary work indicated that the nucleic acid of the H-1 virus is double-stranded DNA while May *et al.* (15), using what appears to be a low concentration of RV, concluded that this agent also contains double-stranded DNA. In view of this it is tempting to speculate that if the nucleic acid of the 9HV-B should prove to be single-stranded, then the 9HV-A may contain the double-stranded type derived from the replicative form of the "B" particle. Studies on the characterization of the nucleic acid types of the 9HV-A and 9HV-B are forthcoming and should shed some light on the interactions in tissue culture of the components of the 9HV complex.

The serological and morphological relationships between the two components, and their role in the induction of peliosis hepatis have not been fully worked out as yet. The earlier observation (1) that anti-RV serum neutralized 9HV (9HV complex) does not necessarily imply that the two components are antigenically identical because the extent of neutralization or inhibition of hemagglutination of the 9HV complex by anti-RV serum may depend on the ratio of 9HV-B to 9HV-A in the preparation being used for the tests.

The fact that the 9HV complex was isolated from leukemic rat tissues should also be considered. If the "A" component has a disease-inducing potential *per se* it is conceivable that its expression is suppressed by the presence of the "B" component particularly if the 9HV-A can "activate" the 9HV-B. The possibility that yet another virus is associated with the 9HV cannot completely be discounted in view of the recent isolation of a previously unrecognized heat labile virus from rat mammary tumors (16). Extracts of such tumors had previously induced leukemia from which the 9HV was isolated (1).

Summary. In vitro characterization studies were carried out with the 9H virus (9HV) which is serologically related to rat virus (RV) and was recently isolated from rat leukemias produced by cell-free extracts of chemically induced rat mammary tumors. Experiments utilizing the techniques of density gradient centrifugation and temperature sensitivity revealed the presence in the 9HV preparations of two types of infectious hemagglutinating components, 9HV-A and 9HV-B, which differed in size and heat stability. Both were resistant to lipid solvents and a wide range of pH conditions, successive propagation of the 9HV-A in REL cell cultures resulted in the appearance of the 9HV-B. Further studies are required to determine the mechanism of this phenomenon. The serological and morphological relationships between the two components have not been delineated as yet.

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Skin-Fixing Properties of Papain Digested Rhesus Antihapten Antibody* (33887)

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Many investigators (1–3) have demonstrated that the F_c fragment of rabbit I_gG retains skin-fixing properties following papain digestion. However, attempts to demonstrate skin fixation with the F_c fragments of papain digested human I_gG or guinea pig γS_1-globulin have been unsuccessful (4–5). Hsiao and Putnam (6) demonstrated that the kinetic rate of digestion of I_gG with papain varied greatly within the animal species studied. Their studies also indicated that the 3.5S fraction B was susceptible to further breakdown upon prolonged digestion with papain. Poulik (7) and Grey and Abel (8) demonstrated that prolonged digestion of both human and nonhuman primates I_gG with papain results in the loss of at least one antigenic determinant of the F_c fragment. This new fragment, F'c, was shown to have slower sedimentation properties (2.1S), a greater electrophoretic mobility than the intact F_c fragment, and was detectable by immunoelectrophoresis after 2 hr of incubation with cysteine and papain.

With these studies in mind, we decided to examine the kinetic rate of digestion of specifically purified rhesus monkey (*Macaca mulatta*) antitrinitrophenol (TNP) antibody in an attempt to determine the optimum time of papain digestion that will retain the skin-fixing properties of the F_c fragment.

Materials and methods. Immunoelectrophoresis of specifically purified rhesus anti-TNP antibody before and after papain digestion was run in 0.05 *M* barbital buffer, pH 8.1, $\mu = 0.05$, for 45 min at 150 V and 4 mA/slide. Upon completion of electrophoretic migration upper and lower troughs were cut and filled, respectively, with rabbit antirhesus total globulins and rabbit antirhesus $7S\gamma_2$-globulin.

Rhesus γ-globulin purified by column chromatography on diethylaminoethyl-cellulose in 0. 1 *M* Tris–HCl buffer, pH 8.0, appeared homogenous in the analytical ultracentrifuge (7S peak only) and showed only a single precipitin band by immunoelectrophoresis against rabbit antirhesus total globulins. Rabbit antirhesus $7S\gamma_2$-globulin antisera was prepared by immunizing animals with 10 mg of the purified γ-globulin in complete Freund's adjuvant at weekly intervals for 3 weeks, and were bled 3–6 weeks after the last injection.

A group of eight rhesus monkeys (3–5 kg) were immunized by intravenous injection with 15 mg of protein of picryl-keyhole limpet hemocyanin (TNP-KLH). The TNP-KLH conjugate was prepared as previously described by Rittenberg and Amrkaut (9), and contains 750 moles of TNP/mole of hemocyanin (determined spectrophotometrically). The amount of antihapten antibody was determined by quantitative precipitation (10) using TNP_{28}-bovine serum albumin as the precipitating antigen. Antihapten antibody titers of the sera obtained from the eight immunized rhesus monkeys varied between 0.5–3.4 mg of antibody protein/ml.

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