Proceedings of the National Academy of Sciences
Vol. 66, No. 2, pp. 344–351, June 1970

Leukemia Viruses Associated with Mouse Myeloma Cells*

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Communicated by S. Spiegelman, December 29, 1969

Abstract. Myeloma cells derived from BALB/c and C3H mice show evidence of infection by a murine leukemia virus. The immunoglobulin-producing myelomas secrete an RNA-containing virus with a density of 1.20 to 1.22 gm/cm^3. RNA with a sedimentation coefficient of 74 S in 0.1 *M* sodium chloride has been isolated from secreted virus particles and has a base composition similar to that found for other murine leukemia virus RNA. An intracellular virus particle has been partially purified and has a density of 1.29 to 1.32 gm/cm^3. Both extracellular and intracellular virus particles contain the leukemia virus group-specific antigen.

Ultrastructural characteristics of viruslike particles in the cytoplasm of mouse plasma cell tumors have been described.[1–3] Here we identify the extracellular and intracellular particles from cultures of myeloma cells secreting immunoglobulin, and from spontaneous variants of these cell lines which secrete only the heavy or light chains of immunoglobulins, as viruses related to the murine leukemia viruses.

Materials and Methods. Cell lines: C1 cells were derived from a myeloma of a C3H mouse; the other tumor lines were derived from oil-induced myelomas of BALB/c mice (see Table 1). Cell lines were cloned in the spleen or in agar,[4] and grown as suspensions in fortified Eagle's medium[10] supplemented with 10 to 20% horse serum. All experiments were performed on exponentially growing cultures with doubling times of 18 to 24 hr.

Virus purification: (*a*) **Extracellular virus:** Cells and debris were removed from the medium by two centrifugation steps (150 *g* for 5 min and 12,000 *g* for 10 min). The medium was layered over 2 ml 15% potassium tartrate containing 0.1 *M* Tris-HCl buffer (pH 8.0) and centrifuged in a Spinco SW25.3 rotor at 25,000 rpm for 60 min. The pellet was resuspended in 0.1 *M* Tris-HCl buffer (pH 8.0) and layered over a 4.8-ml gradient of 15 to 60% potassium tartrate containing 0.1 *M* Tris-HCl buffer (pH 8.0),[11] and centrifuged in a Spinco SW65 rotor at 35,000 rpm for 60 min.

(*b*) **Intracellular virus:** Cells were washed twice in NET buffer (0.1 *M* NaCl, 0.05 *M* Tris-HCl (pH 7.4) 0.001 *M* EDTA), collected by low speed centrifugation, resuspended in NET buffer (approx. 10^6 cells/ml), and stored at -15°C until used. After thawing, Nonidet P40 was added to cell suspensions to a final volume of 1%, the solution pipetted vigorously to ensure lysis of all cells, and then centrifuged at 12,000 *g* for 10 min. The supernatant was layered over 2 ml 15% sucrose containing NET and centrifuged for 60 min in a SW65 rotor at 30,000 rpm. The pellet was resuspended in 0.1 *M* Tris-HCl buffer (pH 8.0) and analyzed by tartrate density-gradient centrifugation.

CsCl-equilibrium sedimentation: Virus solutions were layered over 5 ml previously formed CsCl gradients (1.08–1.43 gm/cm^3) and centrifuged for 20 hr at 15°C in a Spinco SW65 rotor at 50,000 rpm.

Radioactive[3] procedures: ^{3}H-uridine (22.8 Ci/mMole), ^{3}H-thymidine (19.0 Ci/mMole), and ^{14}C [methyl] choline (54 mCi/mMole) were purchased from Schwarz BioResearch Inc. ^{32}P-orthophosphate was purchased from New England Nuclear Corp. For assay of virus secretion, ^{3}H-uridine was added at 5 μCi/ml to 5 $\times$ 10^6 cells in 10-ml cultures. After 16-hr incubation at 37°C, radioactively labeled virus was isolated. To obtain ^{32}P-labeled viral RNA, 2 $\times$ 10^7 cells in 100 ml of phosphate-free culture medium containing 10% horse serum and 20 μCi/ml ^{32}P-orthophosphate were incubated for 24 hr, the cells collected by centrifugation, and placed in fresh radioactive medium for another 24 hr. Virus was purified from the pooled media as described above.

To assay radioactivity, gradient fractions were precipitated by the addition of 2 vol of 10% trichloroacetic acid, collected on Whatman GF/C filters, washed with 5% trichloracetic acid, dried, and counted in a scintillation counter.

RNA procedures: Radioactively labeled virus was collected from the tartrate density gradients and diluted with 2 vol of 0.1 *M* Tris buffer (pH 8.0). Nonradioactive carrier RNA (200 μg) plus an equal volume of a detergent mixture containing 12% sodium 4-aminosalicylate, 2% sodium triisopropylnapthalene sulfonate, 2% NaCl, and 12% *n*-butanol was added to the virus solution and shaken gently for 2 min. An equal volume of a mixture containing 90% phenol, 12% *m*-cresol, 0.1% 8-hydroxyquinoline was added and the total mixture shaken for 15 min. After centrifugation (12,000 *g* for 10 min), the phenol phase was reextracted with an equal volume of the detergent mixture. The aqueous fractions were pooled and extracted twice with fresh phenol mixture. RNA was then precipitated with 2 vol of 95% ethanol (−15°C for 2 hr), collected by centrifugation, dissolved in 1 ml of NET buffer, and reprecipitated with ethanol.

^{14}C-labeled RNA was isolated from purified Newcastle disease virus and ^{3}H-labeled 28S and 18S RNA prepared from P3 myeloma cells for use as reference markers in the determination of the sedimentation coefficient of RNA from the myeloma virus. RNA was analyzed by sedimentation in 15 to 30% sucrose density gradients containing NET buffer in a Spinco SW65 rotor at 50,000 rpm for 90 min. RNA base composition analyses were performed as described elsewhere.[12]

Results. Electron microscopy of the myeloma virus: Myeloma cultures containing virus particles include four IgG secretors, P1, P3, MOPC 70A, and C1; an IgA secretor, S194; and variants that secrete abnormal amounts of heavy and light chains (Table 1). Particles are seen in the cytoplasm (Figs. 1*a* and

TABLE 1. *Properties of myeloma cell lines.*

Cell line	Clones	Strain of origin	Immunoglobulin secreted	Intracellular virus (electron microscopy)	Reference
			(*a*) Myelomas		
X5563	C1·18	C3H	IgG	+	4, 5
MOPC 70A		BALB/c	IgG	+	Isolated by M. Potter; the ascites form is a gift of L. J. Old
ADJ PC 5	P1·17	BALB/c	IgG	+	4, 6
MOPC 21	P3·6·2	BALB/c	IgG	+	4, 7
S194	S194/5·3	BALB/c	IgA	+	8
		(*b*) Spontaneous Myeloma Variants			
		Parent line			
	XP1·17	P1·17	L†	+	4
	S194 J	S194/5·3	H*	+	4, 9
	S194 F and O	S194/5	**	+	4

† Only light chain subunit secreted.
* Excess heavy chain subunit secreted.
** Little heavy chain secreted.

b), budding out from the cell surface membrane (Fig. 1*c*), and as extracellular particles (Fig. 1*d*). The extracellular viral particles have a diameter of about 0.1 μm and contain a double membrane structure. Cytoplasmic particles appear as smaller densely staining, doughnut-shaped structures and do not exhibit the outer membrane.

Properties of virus particles: A culture of P3 myeloma cells containing 5×10^6 cells grown for 24 hr secretes enough particles to form a visible band at a density of about 1.22 gm/cm^3 after sedimentation in a tartrate gradient. This band when examined by electron microscopy contains virus particles similar to those observed in intercellular spaces as shown in Figure 1. These virus particles can be labeled with radioactive precursors of RNA, protein (data not shown), and lipid, but not with a radioactive precursor of DNA (Figs. 2*a* and *b*). Cultures of P1, C1, S194, and XP1 cells also secrete virus particles similar to those isolated from P3 cells.

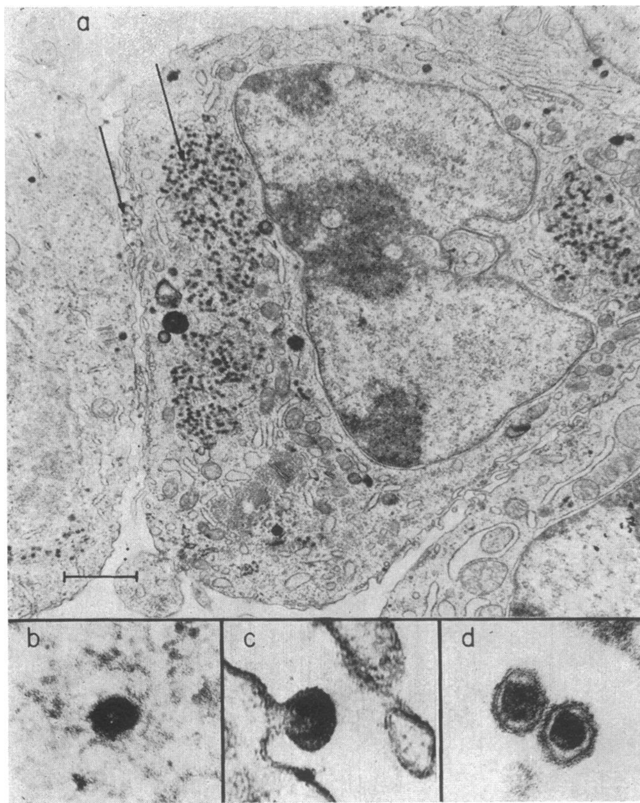

FIG. 1.—Electron micrographs of the virus particles found in P3 myeloma cells. Washed cells were collected by centrifugation, fixed in osmium tetroxide, embedded in Vostopol-W, sectioned, and strained with uranyl acetate and lead citrate. (*a*) View of a single cell showing viral particles in the cytoplasm and secreted virus in the extracellular spaces (*arrows*). The bar represents 1 μm. (Magnification $\times$22,000) Electron micrographs at 10$\times$ higher magnification of (*b*) intracellular, (*c*) budding, and (*d*) extracellular virus. ($\times$220,000) (Courtesy of Marlene Bajak.)

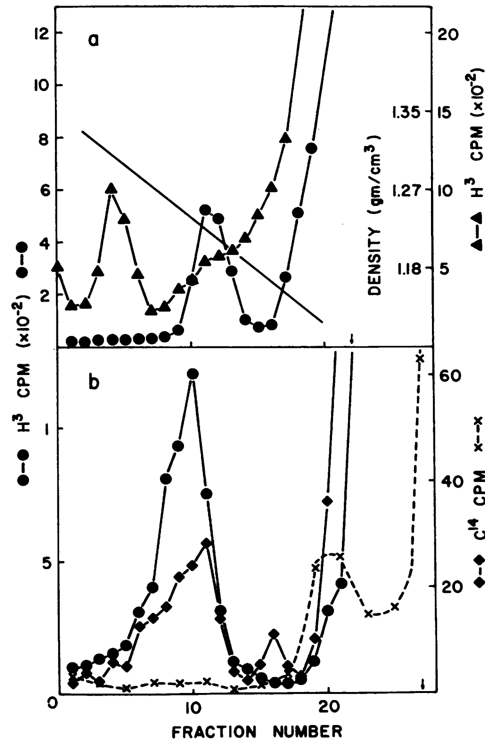

FIG. 2.—Virus purification by tartrate gradient centrifugation. (*a*) 2×10^7 P3 cells in 60 ml medium were labeled with ^{3}H-uridine (5 μCi/ml) for 20 hr. Extracellular virus was purified from the medium and intracellular virus prepared from cell homogenates (*Materials and Methods*). Each virus preparation was sedimented in a 15–60% tartrate gradient for 60 min at 36,000 prm. About 0.1% of total radioactivity incorporated into RNA was found as extracellular viral RNA; 1–2% of radioactivity was associated with the intracellular virus fraction. ●—● Extracellular virus and ▲—▲ intracellular virus. The solid line represents the densities in the previously formed gradient.
(*b*) 1.4×10^6 P3 cells in 5 ml medium were labeled for 16 hr with either ^{3}H-uridine (5 μCi/ml) ●—● and ^{14}C-choline (1 μCi/ml) ◆—◆, or with ^{3}H-thymidine (5 μCi/ml) X—X. Extracellular virus was then purified and centrifuged on a tartrate density gradient as in Fig. 2*a*. Gradient fractions of the virus preparation labeled with ^{3}H-thymidine were incubated with 0.5 *N* KOH at 37°C overnight to hydrolyze RNA. All fractions were then assayed for acid precipitable radioactivity. 0.6% of incorporated ^{14}C-choline, 0.19% of ^{3}H-uridine, and <0.005% of ^{3}H-thymidine in DNA appeared in the virus band.

Intracellular particles appear at a more dense position than extracellular virus after tartrate gradient centrifugation (Fig. 2*a*). This preparation appears to also contain cellular membranes by electron microscopy and attempts to remove these contaminants using various detergents (see below) resulted in the breakdown of the virus particles.

The density of the extracellular virus from P3 cells was 1.20–1.22 gm/cm^3 (Fig. 3*a*), and intracellular virus was 1.29–1.33 gm/cm^3 (Fig. 3*b*), as determined by CsCl-equilibrium sedimentation. These values are similar to the densities determined by the tartrate-gradient centrifugation (Fig. 2*a*). The lower density of the extracellular particle is probably due to its lipoprotein outer membrane,[13, 14] which is not seen in the intracellular particle (cf. Figs. 1*b* and *d*).

Detergent treatment: Detergents were utilized to convert the extracellular virus into particles of higher density by removing the outer lipoprotein membrane.[13, 15] Extracellular particles were isolated from cultures of P3 cells labeled with ^{3}H-uridine and incubated with 1% Nonidet P40. Treatment for 5 min at 4°C did not alter the density of the virus particle. Detergent treatment for 20 min resulted in the loss of labeled virus, with some conversion to a particle with the density of the intracellular virus (Fig. 4). Treatment of extracellular virus with deoxycholate, ether, or Tween 80-ether as described for Rauscher leukemia virus,[15] or with Freon[16] resulted in the loss of acid-precipitable radioactivity from the virus.

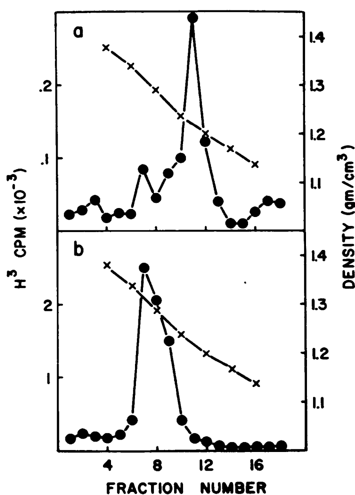

FIG. 3.—Density determination by CsCl centrifugation. ^{3}H-uridine labeled extracellular and intracellular virus particles were prepared as described for Fig. 2*a*. The virus peaks were separately collected and diluted with 1 vol of 0.1 *M* Tris buffer (pH 8.0). 0.2 ml of each virus preparation was layered onto separate previously formed CsCl gradients and centrifuged at 50,000 rpm for 20 hr at 15°. Gradient fractions were collected, density measurements made with a refractometer, and radioactivity determined after acid precipitation. (*a*) Extracellular virus, and (*b*) Intracellular virus.

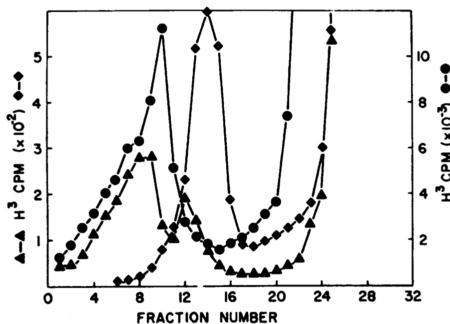

FIG. 4.—Detergent treatment of extracellular virus. A culture containing 3.6 × 10^7 P3 cells in 30 ml medium was labeled for 16 hr with ^{3}H-uridine (5 μCi/ml) and extracellular and intracellular virus was prepared for tartrate-gradient centrifugation. The extracellular virus was resuspended in 0.4 ml 0.1 *M* Tris buffer. Nonidet P40 was added to a 200-μl aliquot of this virus solution to a final concentration of 1% and incubated at 4°C for 20 min. 0.2-ml aliquots of untreated extracellular virus ◆—◆, Nonidet P40-treated extracellular virus ▲—▲, and intracellular virus ●—● were layered on separate tartrate density gradients and analyzed as in Fig. 2*a*. The data have been plotted on one graph.

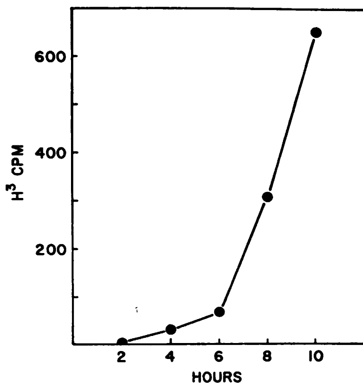

FIG. 5.—Kinetics of virus secretion. Five cultures of P3 cells were incubated with ^{3}H-uridine as for Fig. 2*a*. At each time indicated, the radioactive virus in the extracellular medium of one of the cultures was determined as in Fig. 2*a*. The total radioactivity found in each purified virus fraction has been plotted.

Kinetics of virus release: The appearance of radioactively labeled viral RNA in secreted virus is shown in Figure 5. This is detected as early as 4 hr after the addition of ^{3}H-uridine, and accumulates approximately linearly from 6 to 24 hr. At 18 to 24 hr, the RNA in extracellular virus represents about 0.1% of the total labeled cellular RNA.

Viral RNA: RNA labeled with ^{32}P-orthophosphate was isolated from purified extracellular virus particles. The RNA separated into two components in sucrose gradients containing 0.1 *M* NaCl (Fig. 6*a*). The sedimentation coefficient of the rapidly sedimenting RNA component is about 74 S as determined by using ^{14}C-labeled Newcastle disease

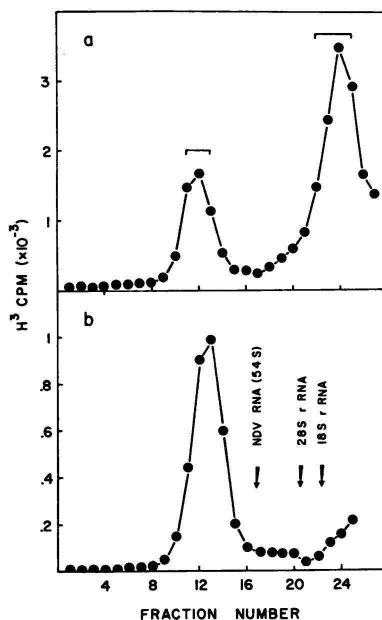

FIG. 6.—Sedimentation of RNA purified from virus. RNA was prepared from ^{32}P-labeled extracellular virus as described in *Materials and Methods*.

(*a*) The total RNA purified from virus was sedimented in a 15–30% sucrose gradient containing NET buffer in a Spinco SW65 rotor at 50,000 rpm for 90 min. The fast- and slow-sedimenting components indicated were separately pooled and reprecipitated with 2 vol of 95% ethanol and 100 μg carrier RNA. Aliquots of each RNA component were used for base composition determinations (Table 2).

(*b*) An aliquot of the ^{32}P-labeled RNA from the rapidly sedimenting component was rerun in another sucrose gradient for the determination of sedimentation coefficients. Separately prepared ^{14}C Newcastle disease virus RNA and ^{3}H 28S and 18S ribosomal RNA were run in separate identical gradients as markers. All of these gradients contained 15–30% sucrose in NET buffer (total vol 4.8 ml). 100-μl RNA in NET buffer was layered on each gradient and sedimented as in (*a*).

virus RNA (54 S in 0.1 *M* NaCl) and ^{3}H-labeled 28S and 18S ribosomal RNA as sedimentation markers (Fig. 6*b*).

The base compositions of the two RNA components isolated from purified virus (marked in Fig. 6) are presented in Table 2. The base composition of the 74S RNA component is similar to that reported for other murine leukemia viruses.[17-20] The base composition of the low molecular weight RNA component was different from that of the 74S RNA component (Table 2), approximating that of mouse cytoplasmic RNA (unpublished data).

Virus negative myeloma: Schubert and Cohn[21] describe a myeloma cell variant, XCl, which does not synthesize immunoglobulin protein. We have observed no viral particles in this cell line by electron microscopy or by radioactive labeling procedures.

TABLE 2. *Base composition of RNA components isolated from virus.*

RNA component	Percentage of Base Composition C	A	G	U
74 S	25.4	24.0	27.5	22.9
Low molecular weight RNA	28.9	19.2	34.8	17.0

Base compositions of ^{32}P-labeled RNA as determined by alkaline hydrolysis and paper electrophoresis of RNA fractionated by sucrose-gradient sedimentation (Fig. 6*a*).

Discussion. We invariably find viruslike particles in a number of myeloma cell lines adapted to tissue culture, including IgG and IgA secretors and variants that secrete only the heavy or light chain subunits of immunoglobulins.

The particles (Fig. 1) are morphologically similar to the murine leukemia viruses.[11, 14, 15, 22, 23] In the cytoplasm, particles appear as densely staining doughnut-shaped structures (Fig. 1*b*). The extracellular particles (Fig. 1*d*)

are secreted by budding[14] from the cell surface (Fig. 1*c*), and in this process the virus obtains an outer membrane not associated with intracellular particles.

The virus secreted by the myeloma cell lines can be radioactively labeled with precursors of RNA, protein, and lipid but not DNA (Fig. 2*c*), as has been found for the murine leukemia[15, 24–26] and sarcoma viruses.[17, 27] Purified, extracellular virus particles have a density of 1.20 to 1.22 gm/cm^3 (Fig. 3*a*). This is higher than the density of 1.16 to 1.18 gm/cm^3 reported for other leukemia viruses (Rauscher,[25] Gross,[11] Moloney, and radiation leukemia viruses,[28] erythroblastosis virus,[27] and the mouse[26] and rat[17] leukemia-sarcoma complex), but is similar to that reported for particles isolated from solid myeloma tumors.[2]

Intracellular virus particles have a density of 1.29 to 1.33 gm/cm^3 (Fig. 3*b*). The extracellular particles could be partially converted to this increased density by incubating with Nonidet P40 for short periods of time (Fig. 4). The increase in density is probably due to the removal of the outer virus membrane as has been reported for detergent-treated Rauscher virus.[13, 15] Treatment with detergents appears to degrade the virus and limits the purification of intracellular particles from cytoplasmic membranes.

The RNA from purified virus has two components (Fig. 6*a*). The high molecular weight component has a sedimentation coefficient (74 S in 0.1 *M* NaCl, Fig. 6*b*) and a base composition (Table 2) similar to that obtained for other leukemia and sarcoma viruses.[17–20, 24, 27] The base composition of the low molecular weight RNA is different from the 74S RNA (Table 2) and does not appear to be a degradation product of viral RNA. The low molecular weight component may be a contamination from the host cell.[27]

The antigenic properties of the myeloma cells described here are discussed in detail elsewhere.[29] Close to 100% of cells of myeloma lines which carry the virus particles are leukemia type G (Gross) positive by the direct cytotoxic test. In addition, both purified extracellular and intracellular virus particles prepared from P3 myeloma cells contain the leukemia group-specific antigen.[29]

On the basis of morphology and physical, chemical, and antigenic properties, we identify the particles observed in the myeloma cells described here as viruses belonging to the murine leukemia-sarcoma group and probably closely related to Gross virus. Attempts to detect infectivity of this particle with tissue culture lines and various strains of mice and rats (newborn and adult) have consistently given negative results when assayed for the synthesis of viral RNA, virus-induced antigens, or other evidence of leukemia.

The initial interest in studying the properties of this virus stemmed from a class of spontaneous variants isolated earlier[5, 21] which appeared to have lost simultaneously the ability to synthesize immunoglobulin, the virus-specific antigens, and the morphologically distinct C-type particles. Variants isolated from three independent myeloma tumors behaved in a similar way[5] suggesting a direct relationship between the two sets of functions. The loss of both the leukemia surface antigen and C-type viral particles has been reported[30] for some rat lymphomas induced by the radiation leukemia virus. We are now pursuing another approach that is to start with an immunoglobulin secreting myeloma line and systematically derive variants lacking the leukemia antigens then score

them for the ability to synthesize and secrete immunoglobulin as unselected markers. Preliminary results indicate that in some of the variants, virus-induced antigens (cell surface antigen G and viral group-specific antigen) and the synthesis of immunoglobulin is simultaneously reduced to the borderline of detection.[29]

We thank Dr. K. Horibata for providing myeloma cell lines; Drs. R. Hyman and G. Grant for unpublished results; Marlene Bajak for the electron microscopy; Margaret S. Hauschka for skillful assistance; and Drs. R. J. Huebner and J. W. Hartley for the typing for the virus antigens.

* Supported by National Cancer Institute contract PH-43-67-1147 and by American Cancer Society, California Division, grant to S. S., also a National Institute of Allergy and Infectious Diseases research grant and a National Cancer Institute training grant to M. C.

† Recipient of Public Health Service fellowship 1 FO2 A138301-01.

‡ Dernham Senior Fellow (D-127) of the American Cancer Society, California Division.

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