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Hamster-Tropic Sarcomagenic and Nonsarcomagenic Viruses Derived from Hamster Tumors Induced by the Gross Pseudotype of Moloney Sarcoma Virus*

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Abstract. Hamster sarcomas induced by the Gross pseudotype of Moloney sarcoma virus yielded a virus sarcomagenic for hamsters but not mice. This virus was able to produce foci on hamster embryo cells, but not on mouse embryo cells. A hamster-tropic nonfocus-forming helper virus was also found in the viral stocks. These hamster-tropic viruses are not immunologically related to the murine viruses in the original inoculum but appear to represent indigenous C-type RNA viruses of the hamster.

Three strains of murine sarcoma viruses, Harvey (H-MSV), Kirsten (Ki-MSV), and Moloney (M-MSV), have been shown to induce sarcomas in hamsters which yield virus oncogenic for hamsters but not for mice. Electron microscopic examination of these hamster tumors reveals characteristic C-type particles.[2–6] The "hamster-tropic" Ki-MSV was also shown to induce foci on hamster cells and not on mouse cells and to be antigenically distinct from its murine precursor virus.[3, 4] Cocultivation of cell lines derived from these virus-induced hamster tumors with mouse embryo fibroblasts and appropriate murine leukemia viruses yielded pseudotype sarcoma viruses with host range and pathologic characteristics indistinguishable from the three murine precursor sarcoma viruses; and in the case of the Ki-MSV gave a virus that again had the murine envelope and group-specific antigens.

This paper describes studies of another "hamster-tropic" sarcoma virus. This virus was isolated from hamster tumors induced originally by the Gross pseudotype of murine sarcoma virus, MSV(GLV), and is designated for the purposes of this report MSV(GLV)(O-H).[7]

Materials and Methods. Viruses: The source of MSV(GLV)(O-H) was a tumored hamster from the fourth passage of a hamster tumor transplant line originally induced by MSV(GLV).[2] Viral concentrates[8] of this tumor induced tumors in hamsters from which a cell-free passage line was established. Concentrates from this tumor line or concentrated tissue culture fluids from viral shedding tumor explants were used as a virus source. Tissue culture grown MSV(GLV) was used in the neutralization test (see as follows). AKR virus was obtained from supernatant fluids of a tissue culture line derived from a lymphosarcoma induced in rats by a wild-type virus isolated from AKR mice.[9]

Animals: Syrian hamsters and NIH Swiss mice were obtained from the National Institutes of Health animal production colony. The LSH, LHC, and MHA inbred strains of Syrian hamsters were obtained from the Lakeview Hamster Colony, New Field, N.J.

Tissue culture: Tissue culture lines were established from tumor explants using Eagle's minimal essential medium with 10% unheated fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). For the focus assays, secondary hamster embryo fibroblasts and mouse embryo fibroblasts were prepared according to published methods.[9, 10] Petri dishes (60 $\times$ 15 mm) (Falcon Plastics) were planted (3.5 $\times$ 10^5 cells/dish) in 4 ml of the medium. Cells were infected with 0.4 ml viral dilution 6–24 hr after plating. The medium was changed every 2–3 days. Foci were counted as early as possible, usually on day 7. *In vitro* rescue experiments were done according to published methods.[11] HT-1[12] cells (8 $\times$ 10^4) were cocultivated with 8 $\times$ 10^5 of either tumor cells shedding MSV(GLV)(O-H), or hamster embryo cells previously infected with dilutions of MSV(GLV)(O-H) including those beyond the focus-forming endpoint. Culture fluids and freeze-thawed cell suspensions were assayed for focus-inducing virus after 15 days of cocultivation.

Complement fixation and immunodiffusion tests: Three known murine leukemia virus antisera were tested in complement-fixation and immunodiffusion tests against antigen preparations of MSV(GLV) (O-H): serum from Fisher rats bearing M-MSV transplant tumors containing complement-fixing antibody to the murine leukemia virus group-specific antigen and envelope antigens; serum from Fisher rats bearing an AKR lymphosarcoma containing complement-fixating antibody to Gross type (G+) murine leukemia virus envelope antigen only; and serum from guinea pigs hyperimmunized with isoelectrofocus purified murine leukemia virus group specific antigen.[13, 14]

Antigens were prepared from tissue culture fluids as highly concentrated viral bands in sucrose density gradients or as highly-concentrated viral pellets. The virus content of different banded preparations was estimated from absorbance determinations at 280 and 260 mμ using a Beckman spectrophotometer. Antigens were also prepared from tumors as either "Moloney procedure" concentrates[8] or as 20% w/v homogenates. Complement-fixation tests were performed by the Microtiter procedure as described previously.[15] Immunodiffusion tests using ether treated antigens[16] were performed on microslides using 0.8% agarose.

Neutralization test: Antisera obtained from guinea pigs by hyperimmunization with MSV(GLV)(O-H) and AKR virus (the latter known to have the murine Gross (G+) type envelope antigen), respectively, were used in the neutralization test. The latter serum completely neutralized 50 focus-forming units of MSV(GLV) at a 1:80 dilution. The viruses used were MSV(GLV)(O-H) and MSV(GLV) at focus-forming titers of 50/0.4 ml and 60/0.4 ml, respectively. Dilutions of inactivated (56°C, 30 min) serum were mixed with an equal volume of virus dilution and kept at 37°C for 30 min; 0.4 ml of the mixture was then inoculated into hamster embryo fibroblasts and mouse embryo fibroblasts tissue culture.

H^3-uridine labeling procedure: The procedure described by Duesberg and Robinson[17] was used to detect and quantitate viral shedding from tumor cell lines and infected hamster embryo cells. Cultures were incubated with 20 μCi uridine H^3 (20 Ci/mmole) per milliliter for 48 hr. Supernatant fluids were then collected, clarified at 10,000 rpm for 10 min, and layered on a 15–60% sucrose gradient with a superimposed zone of 10% sucrose in Tris buffer, pH 7.4. Tubes were centrifuged at 24,000 rpm for 3 hr in the Spinco SW 25.1 rotor or 30,000 rpm for 90 min in the SW 41 rotor (depending on volume). After centrifugation, 0.2 ml-fractions were collected dropwise from the bottom of punctured tubes, precipitated with equal volumes of 10% trichloroacetic acid, collected on millipore filters, and counted in a liquid scintillation counter.

Electron microscopy: Electron microscopic observations were made by Mr. John Walker, Flow Laboratories, using the thin section technique on material which had been fixed in glutaraldehyde and osmium tetroxide, and embedded in a mixture of Epon

812 and araldite and double stained with uranyl acetate and lead citrate. Sections were examined using an Hitachi HU IIE at a scanning magnification of 25,000.

Results. In vivo passage: Newborn hamsters were inoculated with 0.05 ml of a cell-free viral concentrate of a tumor from the fourth hamster transplant passage of the original MSV(GLV) induced hamster tumor (this original hamster tumor occurred after a latent period of 280 days). The tumor incidence was 10 per cent (1/10) and 40 per cent (4/10) at days 75 and 87, respectively, in the first cell-free passage from hamster-to-hamster. With subsequent cell-free passage, tumors were induced progressively more rapidly (Table 1) and by passage four, 27 of 40 inoculated animals had tumors at day 11. Viral concentrates from these passage materials have not induced tumors in 48 NIH Swiss mice infected as newborns and observed for over 100 days.

The tumors arose at or near the site of inoculation and were rapidly growing rhabdomyosarcomas which replaced the leg muscles and often extended to the back and psoas muscles. The tumors were solid but often showed necrotic hemorrhagic areas. Viral concentrates from supernatant fluids of tumor explants also regularly-induced sarcomas in newborn hamsters.

Electron micrographs of the tumors, spleens, livers, kidneys, and lymph nodes of tumored hamsters consistently revealed C-type particles in the tumors and spleens; however, the other tissues were generally negative.

Focus formation by MSV(GLV)(O-H): Viral preparations of MSV(GLV)-(O-H) have induced foci on hamster embryo fibroblasts but not on mouse embryo fibroblasts. The foci were morphologically very similar to those produced by murine sarcoma viruses on mouse cells,[10] containing two types of altered cells: round cells and spindle cells that differed from normal fibroblasts in that they were thinner and more refractile. Piling up and criss-crossing of cells was noted, but was not a conspicuous feature (Fig. 1). Foci became observable by day 6 or 7. They enlarged slightly over the next two to three days and then became stationary. A few additional foci were first noted on days 8 and 9. These were indistinguishable from and not disproportionately localized near earlier appearing foci and thus were thought to also represent primary foci.

Focus titration curve: Viral concentrates of tumors from passage 3 were titrated on hamster embryo fibroblasts of the LSH strain as these cells were found to be more sensitive than the other three strains tested.[18] Serial twofold

TABLE 1. *Cell free transmission of MSV(GLV)(O-H) in hamsters.*

Passage no.	Tumors/survivors (days postinoculation)
Initial inoculation MSV(GLV)*	1/17 (280), 3/17 (339)
1†,‡	1/10 (75), 4/10 (87)
2	4/10 (33)
3	12/16 (20), 15/16 (32)
4	27/40 (11)

* Inoculations consisted of 0.05–0.1 ml of 1 gm equivalent "Moloney procedure" tumor concentrates. Four viable cell passages between the initial tumor and passage 1.

† This and subsequent passage materials have not induced tumors in mice over a 200-day observation period.

‡ This and subsequent passage materials produce typical foci of transformed cells in hamster embryo cells but not in mouse embryo cells.

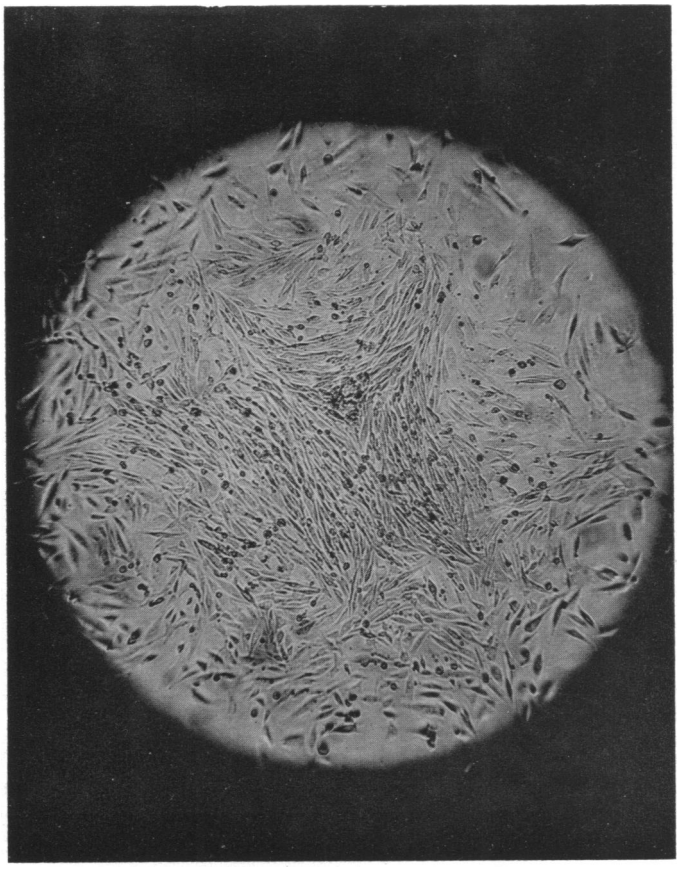

FIG. 1.—A typical focus of transformed cells induced by MSV(GLV) (O-H) on hamser embryo fibroblasts.

dilutions were prepared and distributed to each of five plates. This made it possible to determine with confidence the relationship between focus count and dilution, i.e., one-hit versus two-hit kinetics. The results clearly show that focus formation by this particular virus preparation followed one-hit kinetics (Fig. 2). This is not at present interpreted as evidence for a nondefective sarcoma virus since similar results can be obtained if either focus production does not require viral replication[19] or a great excess of helper virus was present. Based on evidence presented below, it appears clear that a nonfocus-forming virus is present in excess in MSV(GLV)(O-H) stocks; however, this virus is present in only approximately 10- to 100-fold excess and thus cannot account for the one-hit curve.

Presence of a nonfocus-forming virus in viral stocks: Viral shedding (revealed by the ^{3}H-uridine labeling technique) from tissue culture plates that had received a dilution of virus tenfold higher than the focus-forming titer suggested that a nonfocus-forming virus was present in the stocks of MSV(GLV)(O-H). These cultures were also shown to contain characteristic C-type RNA virus particles by electron microscopy. Supernatant fluids and virus suspensions from

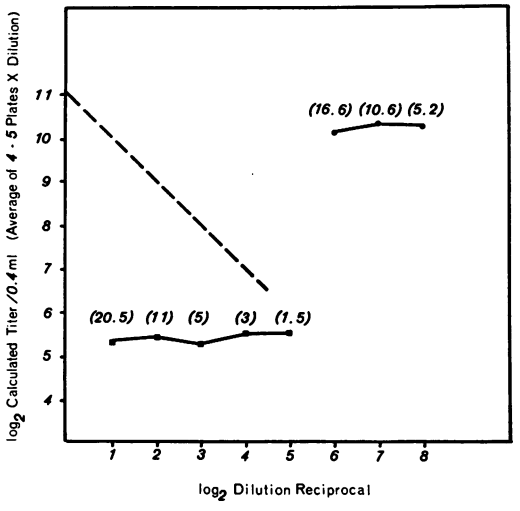

FIG. 2.—Kinetics of focus formation by MSV-(GLV) (O-H). Serial 2-fold dilutions of a passage 3 tumor preparation (●—●) and supernatant fluid from a tumor explant (■—■) were plated on hamster embryo fibroblasts of the LSH strain. Figures in parentheses are averages of focus counts of 4–5 plates per dilution. The slope of a theoretical 2-hit curve is shown (broken line).

freeze-thawed cells of this culture have not shown focus-forming activity even after several passages; however, these cultures remain positive by electron microscopy and still shed amounts of virus particles equivalent to that obtained from plates that have foci and yield focus-forming virus.

In repeated experiments using electron microscopy, H^3-uridine labeling, and specific interference with focus formation as criteria, the sarcomagenic virus stocks contain 10- to 100-fold more nonfocus-forming virus than focus-forming virus.

Evidence of helper function of the nonfocus-forming virus: Cocultivation for 15 days of the culture shedding only nonfocus-forming virus, mentioned previously, with HT-1 cells resulted in viral harvests that had focus-forming activity on hamster embryo cells but not on mouse cells (Table 2). This was interpreted as positive evidence of a helper function of the nonfocus-forming virus. Successful rescue of the MSV genome from HT-1 cells was also achieved by cocultivating them with tumor cell lines of MSV(GLV)(O-H) shedding both focus-forming and nonfocus-forming virus. The resulting viral harvest had a much higher focus-forming titer than the harvest from the control plates containing only tumor cell lines. These results provide evidence that the nonfocus-forming virus is a hamster-specific helper virus.

Immunologic evidence for a new virus(es): Viral concentrates (1000 fold, v/v after pelleting and gradient purification) from supernatant fluids obtained from virus shedding cultures of MSV(GLV)(O-H) and AKR-induced tumors

TABLE 2. *Rescue of the defective murine sarcoma virus genome by the nonfocus-forming virus in MSV(GLV) (O-H) stocks.*

	Cell Lines Tested for Focus-Forming Activity		
	GLV(O-H)*	HT-1†	GLV(O-H) + HT-1
Focus-forming units/ml on hamster embryo fibroblasts	0	0	110‡
Focus-forming units/ml on mouse embryo fibroblasts	0	0	0

* Cell lines producing nonfocus-forming virus derived from cells producing C-type virus beyond the MSV(GLV)(O-H) focus endpoint.

† Nonproducer hamster tumor cell line produced by M-MSV.[11, 12]

‡ Focus-forming units/ml of culture harvests.

TABLE 3. *Absence of murine leukemia envelope and group specific antigens in MSV(GLV)-(O-H).*

Virus*	Protein concentration (mg/ml)	Test Sera§					
		MSV Rat		AKR Rat		Guinea Pig Anti Group Specific	
		Fresh†	Ether‡	Fresh†	Ether‡	Fresh†	Ether‡
AKR	0.35	128‖	128	64	8	<4	128
M-MSV(GLV)(O-H)	0.40	<2	<2	<2	<2	<2	<2

* Purified by density gradient centrifugation from tissue culture supernatant fluids. These preparations contain 4–5% nucleic acid based on absorbance values at 280 and 260 mμ. Protein concentrations were calculated by the method of Warburg and Christian.[20]

† Preparations obtained from density gradients tested immediately.

‡ Ether treated according to Geering *et al.*[16]

§ Extensive complement-fixation tests with these sera indicate that "MSV rat" reacts with envelope and group specific antigens of all murine C-type viruses,[21] AKR rat is relatively specific for the Gross subgroup envelope antigen, guinea pig anti-gs prepared against highly purified antigen (isoelectric focused) reacts only with the murine group specific antigen.

‖ Reciprocal of complement-fixation titer using 4 units of indicated serum.

were tested in the complement-fixation and immunodiffusion tests against three murine leukemia virus antisera whose specificities are shown (Table 3). The results indicate at least a 50-fold difference in titer of murine leukemia virus antigens per microgram of virus protein between AKR virus and MSV(GLV)(O-H). Ether disrupted virus produced precipitin lines in immunodiffusion tests for the group specific antigen of AKR virus while no reactions were obtained with MSV-(GLV)(O-H). Similar results were obtained with antigens from the hamster tumor preparations.

When screened at a 1:10 dilution guinea pig antiserum prepared against purified MSV(GLV)(O-H) neutralized the focus-forming activity of the virus significantly and did not neutralize MSV(GLV). Conversely, guinea pig antisera prepared against AKR virus and known to completely neutralize 50 focus-forming units of MSV(GLV) at a 1:80 dilution failed to neutralize MSV(GLV)(O-H) (Table 4).

The degree of difference in the amount of murine leukemia virus antigens in the two viruses and the failure of crossneutralization is interpreted as evidence that MSV(GLV)(O-H) is not merely a "hamster-adapted" murine virus, but should be considered a "new" virus(es), most probably of hamster origin. Further evidence for this comes from our recent findings that hamsters bearing MSV(GLV)-(O-H) tumors produce virus neutralizing antibody. Such sera and the guinea pig antisera reported above also neutralized the hamster-tropic viruses derived from other murine virus induced hamster tumors. This data along with results of *in vitro* interference experiments[22] indicates that the hamster-tropic viruses have a common envelope; however, reciprocal tests among these isolates will be necessary to firmly establish this hypothesis.

TABLE 4. *Antigenic differentiation of MSV(GLV) and MSV(GLV)(O-H).*

Test virus	Antisera*	
	AKR	MSV(GLV)(O-H)
MSV(GLV)	100%†	0
MSV(GLV)(O-H)	0	97.5%

* Sera used at 1:10 dilution.

† Degree of focus reduction with input virus levels of 40–50 focus-forming units/test on several trials.

Discussion. The studies reported here on MSV(GLV)(O-H) are generally in agreement with studies on H-MSV(O-H), KiMSV(O-H), and M-MSV(O-H)[3-6] and further provide evidence for a nonfocus-forming helper virus in the hamster-specific sarcoma virus preparations. The absence of murine leukemia virus antigens in concentrates of MSV(GLV)(O-H) as compared to similar concentrations of AKR virus, and the lack of crossneutralization with appropriate antisera is unequivocal evidence that MSV(GLV)(O-H) is at least antigenically a "new" virus(es). However, since the only measurable differences between MSV(GLV)-(O-H) and MSV(GLV) are antigenic structure and host range, and since these are probably exclusively helper functions, there is no evidence that the genome of the focus-forming virus in the MSV(GLV)(O-H) stock differs from its focus-forming MSV precursor. Conversely, the ability of the helper virus in the MSV-(GLV)(O-H) stock to serve as a helper for a defective murine focus-forming genome and the pathologic similarity of murine sarcomas induced by H-MSV, Ki-MSV, and M-MSV to those induced by their respective murine pseudotype sarcoma viruses made after passage through hamsters[3, 5, 6] is suggestive evidence that the same sarcomagenic genome can be passed from species to species with the aid of specific helper viruses. Ultimate proof of this may only be obtained when genetic markers of the sarcoma genome are available.

The source of the hamster-specific helper virus is most likely the indigenous C-type RNA virus of the hamster that has been visualized in hamster tumors that occurred spontaneously or were induced by adenovirus or SV40.[23] A C-type virus has also been seen in hamster tumors induced by a hamster papova-like virus; once obtained this C-type virus induced leukemia in hamsters by cell-free passage.[24] Immunologic and biologic studies to establish the relationship between the helper virus reported in this paper and the indigenous hamster C-type virus(es)[23, 24] are in progress. The other possible, but unlikely, sources of the helper virus is that it is a mutant of the input Gross leukemia virus or that it existed as a contaminent in the original virus stocks. The former possibility is untenable in view of the immunologic data obtained here, since the existence of a mutant with noncrossreacting envelope or group-specific antigens would be extremely improbable. The latter possibility can be tested when specific antisera to the MSV(GLV)-(O-H) group specific antigens become available.

Inoculation of a sarcoma virus into a host where its input helper cannot replicate itself readily, or at a high dilution so that it is not accompanied by a helper virus in each cell, can result in an output virus with a marked change in host range and antigenic composition.[3, 5, 6, 25-27] Studies of RSV(O)[25, 26] and MSV-(O)[27] have favored the presence of competent helper-independent sarcoma viruses to explain these changes rather than activation of an indigenous helper virus as suggested here; however, conclusive evidence for lack of a helper virus for RSV-(O) and MSV(O) has not been reported.[25-27] More studies are required to finally establish that the helper virus present in the MSV(GLV)(O-H) stocks is indeed the indigenous hamster C-type virus. In our extensive experience with hamster tumors and normal tissues, we have not found C-type virus shedding cultures similar to those reported here. We tentatively assume that if this new virus is hamster derived, its synthesis was derepressed by the murine sarcoma virus

genome. Our data and that reported for other MSV hamster-specific sarcoma viruses suggest that inoculations of murine sarcoma viruses *in vivo* or *in vitro* into different species may be a generally effective new method of detecting and isolating presumed indigenous C-type RNA viruses in species in which they haven't yet been found.

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[1] Abbreviations: MSV, murine sarcoma virus; H-MSV, Harvey murine sarcoma virus; Ki-MSV, Kirsten murine sarcoma virus; M-MSV, Moloney murine sarcoma virus; MSV-GLV, Gross pseudotype of murine sarcoma virus.

[2] Huebner, R. J., unpublished data.

[3] Klement, V., J. W. Hartley, W. P. Rowe, and R. J. Huebner, *J. Natl. Cancer Inst.*, in press.

[4] Sarma, P. S., T. S. Log, and R. V. Gilden, *Proc. Soc. Exptl. Biol. Med.*, in press.

[5] Bassin, R. H., P. J. Simons, F. C. Chesterman, and J. J. Harvey, *Int. J. Cancer*, **3**, 265 (1968).

[6] Perk, K., M. V. Viola, K. L. Smith, N. A. Wivel, and J. B. Moloney, *Cancer Res.*, **29**, 1089 (1969).

[7] Following the convention of Klement *et al.*, for Ki-MSV and Ki-MSV(O-H): "O" designates that it is no longer "tropic" for its indigenous host, "H" designates that it is "hamster-tropic." Note this convention is different from (0), where (0) is used to designate that no helper virus was detectable in the RSV stocks.

[8] Moloney, J. B., *J. Natl. Cancer Inst.*, **24**, 933 (1960).

[9] Hartley, J. W., W. P. Rowe, W. I. Capps, and R. J. Huebner, these PROCEEDINGS, **53**, 931 (1965).

[10] Hartley, J. W., and W. P. Rowe, these PROCEEDINGS, **55**, 780 (1966).

[11] Huebner, R. J., J. W. Hartley, W. P. Rowe, W. T. Lane, and W. I. Capps, these PROCEEDINGS, **56**, 1164 (1966).

[12] This hamster tumor cell line induced by M-MSV carries the MSV genome but does not produce virus particles or virion antigens. Superinfecting leukemia viruses provide their distinct envelope types to the rescued sarcoma virus. The rescued virus is called a pseudotype virus according to current convention.[11]

[13] Gregoriades, A., and L. J. Old, *Virology*, **37**, 189 (1969).

[14] Oroszlan, S., in preparation.

[15] Huebner, R. J., W. P. Rowe, H. C. Turner, and W. T. Lane, these PROCEEDINGS, **50**, 379 (1963).

[16] Geering, G. L., L. J. Old, and E. A. Boyse, *J. Exptl. Med.*, **124**, 753 (1966).

[17] Duesberg, P. H., and W. S. Robinson, these PROCEEDINGS, **55**, 219 (1966).

[18] Lee, Y. K., unpublished data.

[19] Rubin, H., *Virology*, **10**, 29 (1960).

[20] Warburg, O., and W. Christian, *Biochem. A.*, **310**, 384 (1941).

[21] Hartley, J. W., W. P. Rowe, W. I. Capps, and R. J. Huebner, *J. Virology*, **3**, 126 (1969).

[22] Kelloff, G. J., R. J. Huebner, N. H. Chang, and R. V. Gilden, unpublished data.

[23] Stenback, W. A., G. L. VanHoosier, and J. J. Trentin, *Proc. Soc. Exptl. Biol. Med.*, **122**, 1219 (1966).

[24] Graffi, A., T. Schramm, E. Bender, I. Graffi, K. H. Horn, and D. Bierwolf, *Brit. J. Cancer*, **22**, 577 (1968).

[25] Vogt, P. K., these PROCEEDINGS, **58**, 801 (1967).

[26] Hanafusa, H., and T. Hanafusa, *Virology*, **34**, 630 (1968).

[27] Ting, R. C., *J. Virology*, **2**, 865 (1968).