Effect of Sodium Bicarbonate Upon Myxoma Virus Plaque Morphology. (31253)

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In our studies of the kinetics of myxoma virus replication in various cell lines, assay of virus was made on monolayers of an established rabbit kidney cell line (RK_{13}-glaxo). In the course of these assays, it was observed that the plaque morphology was influenced by the presence or absence of sodium bicarbonate in the agar overlay medium. This paper describes the similarities and differences between myxoma virus plaques produced under these two conditions.

Materials and methods. Virus. The South American Sanarelli strain of myxoma virus was employed in these studies. Stock virus (MV) was prepared from the spleens of infected rabbits. Normal rabbits were inoculated with 0.1 ml of viral suspension containing 1×10^4 RID_{50} (50% rabbit infective dose) at each of 5 spots. When the animals showed signs of generalized myxomatosis, they were sacrificed and their spleens aseptically harvested. Spleens were thoroughly ground with sand by means of a mortar and pestle; large pieces of tissue were sedimented by light centrifugation and the supernatant fluid was diluted to constitute a 20% spleen suspension in tris-lactalbumin hydrolysate-modified Hanks' salt medium. All MV stocks were stored at −70°C.

Cell cultures. The established line of rabbit kidney cells (RK_{13}-glaxo) was propagated in a medium of tris-lactalbumin hydrolysate-modified Hanks' salt (T-L-MH)(1) supplemented with 5% fetal bovine serum (FBS) (Microbiological Associates). The initial seeding consisted of 6.0×10^5 cells in 3-oz prescription bottles. After complete monolayers were formed (7th day after seeding), cells were removed by means of a trypsin-versene solution (TVS) which consisted of 0.05% trypsin and 0.025% versene in saline A(1). These cells were centrifuged and resuspended in T-L-MH medium containing 5% FBS at an appropriate dilution and distributed into new bottles. RK_{13} cells were uniformly epithelial-like in monolayers on glass.

Agar overlay medium. This consisted of 0.5% lactalbumin hydrolysate, 0.1% yeast extract, 0.1% bovine albumin, 6% horse serum and 0.85% Difco agar in modified Hanks' salt solution. When sodium bicarbonate was used, 3 ml of a 7.5% sodium bicarbonate solution was added to 100 ml of the agar overlay medium.

Virus assay. Plaque-count method. This was modified from Padgett *et al*(2). RK_{13} cell monolayers in 3-oz prescription bottles were washed with 2.0 ml of T-L-MH medium; wash fluid was removed and each bottle was inoculated with 0.1 ml of an appropriate virus dilution. The inoculated bottles were incubated at 37°C for 4 hours for adsorption of virus, and the viral inoculum was periodically redistributed over the entire cell sheet by tilting of the bottles several times during the adsorption period. After the adsorption period, the cell sheet in every bottle was covered with 10 ml of agar overlay medium, and the cultures incubated at 37°C. With the RK_{13} cell cultures, plaques usually appeared on the second day after incubation and were easily counted after 3 additional days. No staining of the cell sheet was needed.

Neutralization tests. Equal volumes of appropriate virus dilutions and aged, undiluted normal or immune rabbit serum were mixed and allowed to stand at room temperature for 2 hours. At the end of this period, the numbers of unneutralized viral particles were determined by the plaque method on RK_{13} cell monolayers. The immune serum was obtained from rabbits two weeks after intradermal immunization with 3.0×10^5 plaque forming

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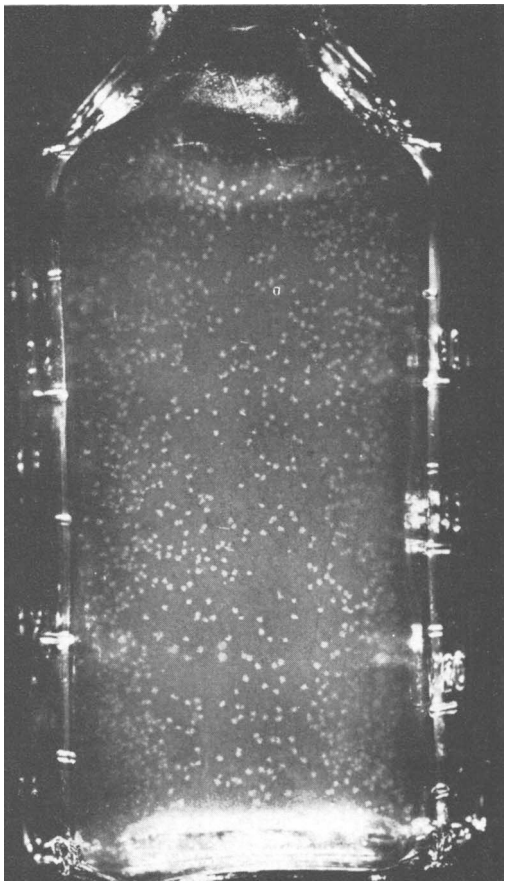

FIG. 1. Myxoma virus plaques on a monolayer of RK_{13} cells in a 3-oz prescription bottle, 6 days after infection, under an agar overlay medium that completely lacked sodium bicarbonate, × 2.

units (PFU) of an attenuated strain of myxoma virus kindly supplied by Dr. D. G. McKercher, University of California, Davis.

Results. Plaque morphology on RK_{13} cells. In the absence of sodium bicarbonate, MV plaques began to appear as very tiny and very opaque dots on the second or third day after inoculation of cultures with virus. They became very distinct and easily seen and counted by the naked eye on the fifth or sixth day after inoculation (Fig. 1). The plaque slowly, but progressively increased in size over a period of at least 20 days. The maximum size of the plaque attained was approximately 2 to 3 mm in diameter. The number of plaques per bottle was, however, stabilized on the fifth day after inoculation. By microscopic examination, no cellular destruction in the plaque area could be detected in the early stages of infection; at this time the plaques consisted of accumulation of cells showing cell morphologies which differed markedly from those of surrounding cells (Fig. 2).

When sodium bicarbonate was incorporated in the agar overlay medium, the initial stages of plaque formation by MV on RK_{13} monolayers resembled those produced by the virus in the absence of sodium bicarbonate. However, on the fourth day after inoculation, microscopic examination revealed that many of these plaques (developing in the presence of sodium bicarbonate) showed some clearing in the plaque area. By the sixth day after inoculation, all plaques were of the new type (*i.e.*, exhibiting cellular destruction as shown in Fig. 3). At this time, the area of cellular degeneration constituted 40% to 70% of the total plaque area. The microscopic change in the morphology of MV plaques on RK_{13} cells in the presence of sodium bicarbonate was associated with a simultaneous change in its gross morphology; as shown in Fig. 4, the plaques now exhibited an opaque, mesh-like appearance.

Specificity of MV plaques. The specificity of the two types of MV plaques obtained as described above was indicated in two ways (results not shown). Firstly, suspensions prepared from these plaques induced fatal myxomatosis disease and characteristic lesions when inoculated intradermally into rabbits. Secondly, the ability of MV to produce plaques on RK_{13} monolayers was inhibited either completely or to a very significant extent by incubation of virus with specific anti-myxoma serum.

The conclusion that despite morphological differences, both types of plaques were produced by myxoma virus is further suggested by the identical number of plaques produced under the two sets of experimental conditions (Table I).

Effect of sodium bicarbonate upon virus yield. One possible explanation for the two types of plaques is that stock viral suspensions contained two forms of myxoma virus; this possibility was eliminated by experiments (results not shown) which indicated that virus from either type of plaque was capable

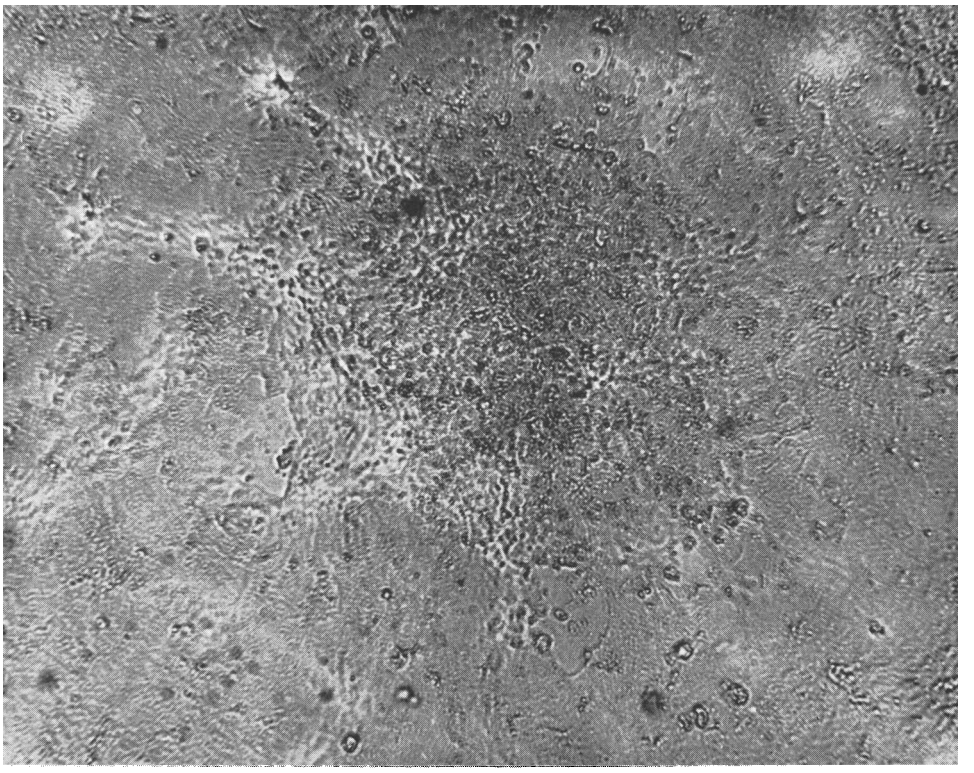

FIG. 2. A myxoma virus plaque area on monolayer of RK_{13} cells as revealed by microscopic examination, 6 days after infection. No sodium bicarbonate present in agar overlay medium, × 128.

of forming either of the two plaque types and that the plaque morphology depended solely upon the presence or absence of sodium bicarbonate in the overlay medium. A second possible explanation of the two plaque morphologies is that sodium bicarbonate may influence the yield of virus from infected cells and that higher yields of virus from cells infected in the presence of sodium bicarbonate may result in demonstrable necrosis of cells and thus in altered plaque morphology. That this was not the case is shown by the results of Table II. It is apparent that RK_{13} cell monolayers infected with myxoma virus and cultured in liquid medium with or without sodium bicarbonate gave identical yields of virus at 19-20 hours and 50-55 hours post infection.

Discussion. The variation in both the microscopic and macroscopic appearance of the myxoma virus plaques produced on RK_{13} cells in the presence and absence of sodium

TABLE I. Comparison of Plaque Numbers in Presence and Absence of Sodium Bicarbonate.

MV stock	$NaHCO_3$*	
	Present	Absent
1	5.4×10^5	3.5×10^5
2	2.3×10^5	2.7×10^5
3	1.5×10^5	1.4×10^5

* 3 ml of 7.5% $NaHCO_3$ per 100 ml of agar overlay medium.

TABLE II. Effect of Sodium Bicarbonate upon Virus Yield from RK_{13} Cells.

Exp No.	Hr post infection*	Yield of virus (PFU/cell culture)	
		Bicarbonate absent	Bicarbonate present
1	19	1.2×10^6	1.0×10^6
	55	2.7×10^6	2.7×10^6
2	20	1.1×10^6	8.8×10^5
	50	2.6×10^6	2.4×10^6

* RK_{13} cell monolayers were infected with virus and incubated in liquid medium (TLMH) with or without sodium bicarbonate. At the designated times, cultures were tested for virus concentration by the plaque assay method.

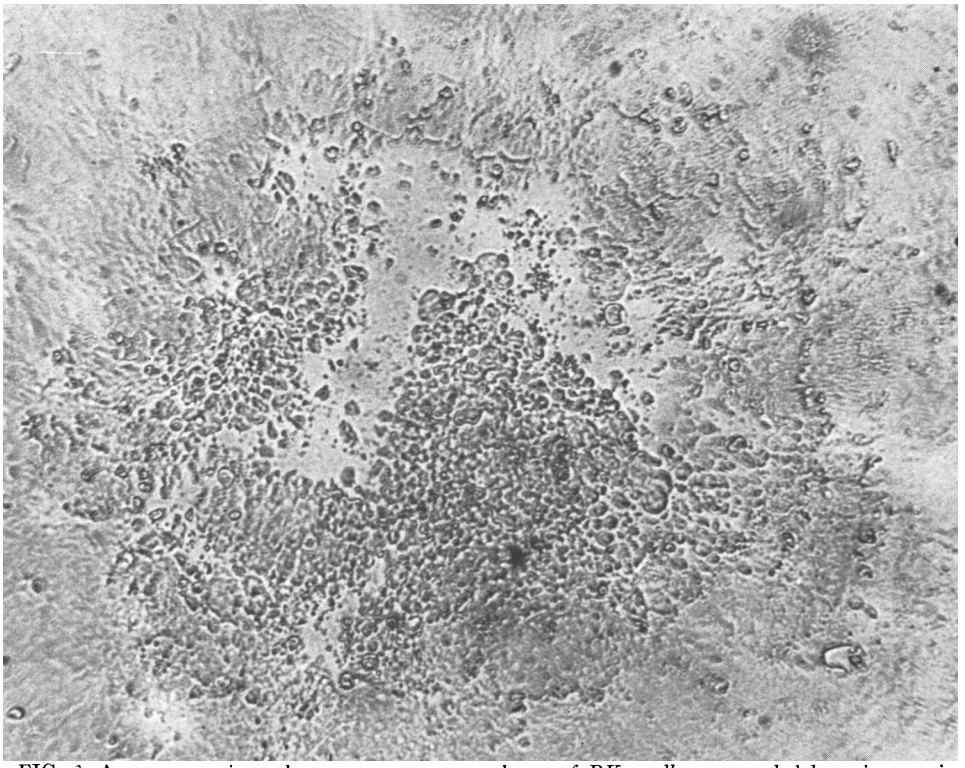

FIG. 3. A myxoma virus plaque area on a monolayer of RK_{13} cells as revealed by microscopic examination, 6 days after infection. Sodium bicarbonate present in the agar overlay medium at a concentration indicated in the text, × 128.

bicarbonate in the overlay medium has not been reported before. The possibility that these two types of plaques were produced by two variants of myxoma virus in our stocks was ruled out by the observation that when myxoma virus isolated from either plaque type was grown on RK_{13} cell monolayers, only one type of plaque was produced (the type of plaque produced depended only upon the presence or absence of sodium bicarbonate in the agar overlay medium). Therefore, it would seem that the effect of sodium bicarbonate on the structure of myxoma virus plaque was of transient nature. The mechanism of this phenomenon is unknown. In view of the fact that myxoma virus is considered by some workers to be a tumor virus, one possibility might be that virus-infected cells are induced to undergo proliferation and that this proliferative tendency is suppressed by certain environmental conditions such as, for example, the presence of sodium bicarbonate. Further work on this point is needed. The present observations are obviously different from those described for attenuated poliomyelitis virus where plaque formation is dependent upon an adequate concentration of bicarbonate(3).

The method of plaque assay of myxoma virus described by Padgett *et al*(2) utilized primary lines of rabbit kidney cells. It would have been of interest to determine whether sodium bicarbonate has a similar effect upon plaques produced in primary cell lines; however in our experience, primary rabbit cell monolayers proved entirely unsatisfactory for plaque studies, because of the consistent occurrence of nonspecific degeneration of cells under the agar overlay. No mention was made by either Padgett and co-workers(2) or Schwerdt and Schwerdt(4) about the development of such degeneration in primary rabbit kidney cells. The basis of this nonspecific degeneration is unknown, but it is interesting

to note that with primary and established rabbit kidney cell cultures, Verna and Eylar (5) developed a plaque assay method for titration of fibroma virus using a liquid growth medium overlay; these workers noted that conventional procedures using agar-containing overlay medium for animal virus plaque assay were unsatisfactory when applied to the fibroma virus due to poor cell maintenance.

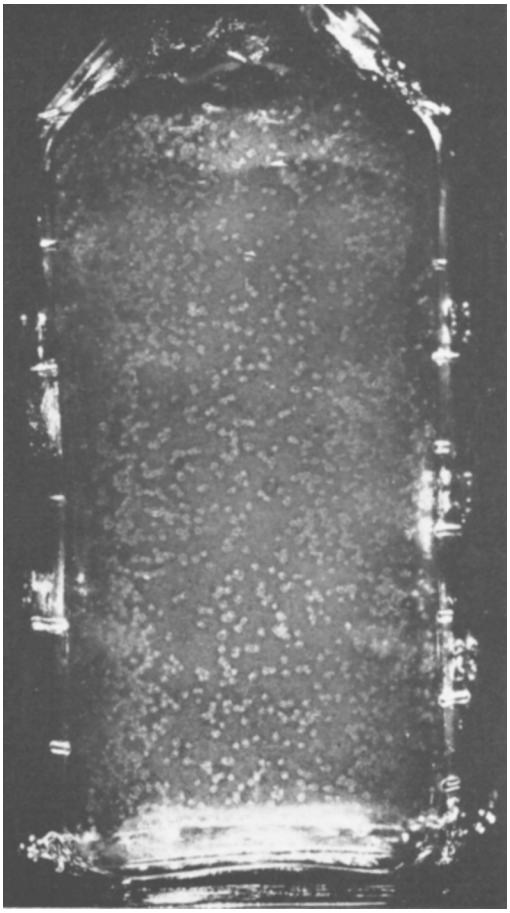

FIG. 4. Myxoma virus plaques on a monolayer of RK_{13} cells in a 3-oz prescription bottle, 6 days after infection, in the presence of sodium bicarbonate in the agar overlay medium, × 2.

Summary. Two morphologically distinct types of MV plaques were produced on monolayers of RK_{13} cells under agar; the appearance of these plaques depended upon the presence or absence of sodium bicarbonate in the agar overlay medium. The numbers of plaques produced by a given suspension of myxoma virus were identical, regardless of the presence or absence of sodium bicarbonate. Moreover, the yield of virus from infected RK_{13} cells was not altered by addition or omission of sodium bicarbonate. The specificity of the two plaque types was established by pathogenicity tests and neutralization of plaque forming units by anti-myxoma serum.

1. Madin, S. H., Andriese, D. C., Darby, N. B., Am. J. Vet. Res., 1957, v18, 932.
2. Padgett, B. L., Moore, M. S., Walker, D. L., Virology, 1962, v17, 462.
3. Vogt, M., Dulbecco, R., Wenner, H. A., ibid., 1957, v4, 141.
4. Schwerdt, P. R., Schwerdt, C. E., Proc. Soc. Exp. Biol. and Med., 1962, v109, 717.
5. Verna, J. E., Eylar, O. R., Virology, 1962, v18, 266.

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Polyacrylamide Gel Electrophoresis: Hormonal and Species Specificity Of Antibody Binding of Bovine I^{131} Thyrotropin.* (31254)

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Previous publications have described the resolution of I^{131} TSH^3(1) and I^{131} insulin (2) from their soluble antibody complexes by means of disc electrophoresis in polyacrylamide gel. Because of the possible usefulness

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