Microepidemiology of Mumps Virus Infection in HeLa Cell Monolayers[1] (36173)

CATHERINE W. C. DAVIS AND JOSEPH W. ST. GEME, JR.
Department of Pediatrics, Harbor General Hospital, UCLA School of Medicine, Torrance, California 90509

Transfer of progeny virus between cells can occur through an extracellular antibody sensitive phase [*e.g.*, Newcastle disease virus (1), poliovirus (2)], through antibody insensitive spread via cell bridges or cell fusion [*e.g.*, vaccinia virus (3–5), herpesvirus (2, 6, 7), measles virus (8)], or passively through division of infected cells [*e.g.*, mumps virus (9), Newcastle disease virus (10), measles virus (11), rubella virus (12, 13)]. As illustrated by the work with herpesvirus (7), study of the microepidemiology of different viruses is important in interpreting viral spread or persistence *in vivo*.

Slow development of mumps virus secondary plaques in a hemadsorption plaque assay in HeLa cell monolayers under a liquid overlay (14) suggested that direct cell-to-cell spread, rather than transfer through the fluid phase, could be the predominant means of spread of this virus, even though no cell fusion was apparent in this system. On the other hand, mumps virus infection of monkey fetuses *in utero* appears to terminate rapidly with appearance of specific neutralizing antibody in the maternal serum, which would suggest that an antibody sensitive phase is important in spread of the virus (15). In chickens infected *in ovo* with mumps virus, infectious virus disappears within 1 week after hatching, even though neutralizing antibody has not been detected in sera until 1 month of age (16). In order to study the effect of neutralizing antibody on the spread and persistence of mumps virus infection, we have studied plaque development in HeLa cell monolayers in the presence and absence of rabbit hyperimmune mumps virus antiserum in the medium.

Materials and Methods. General techniques of cell cultivation and viral assay used in these experiments have been previously described (14). Cells were maintained in Eagle's basal medium using Hanks' balanced salt solution (BSS) and supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and 5% heat-inactivated fetal bovine serum (MM). Hemadsorption was carried out using a 0.1% suspension of guinea pig erythrocytes at 4°.

Mumps virus hyperimmune serum was prepared in rabbits by repeated subcutaneous inoculations of HeLa cell-propagated stock mumps virus, with or without Freund's incomplete adjuvant, after an initial intravenous inoculation of the virus. Serum used in the present experiments neutralized 300 ID_{50} of mumps virus at a dilution of 1:1024, and was diluted 1:10 or 1:15 with MM. It is referred to as "antiserum-MM." Two types of control rabbit serum were used: serum obtained from rabbits prior to virus inoculation, and serum from rabbits which had received multiple subcutaneous inoculations of cell-free medium from cultures of uninfected HeLa cells. These were also used at a 1:10 or 1:15 dilution with MM, and are called "control-MM." All sera were heat-inactivated at 56° for 30 min, except in the experiments involving immune cytolytic plaques (see below).

Experiments evaluating the effect of antiserum on the evolution of mumps virus plaques were complicated by the fact that

[1] This investigation has been supported by a grant from the Los Angeles County Heart Association, General Research Support Grant RR05551 from the National Institutes of Health, and U.S. Public Health Service Training Grant IT01 AI 00 367.

TABLE I. Numbers of Hemadsorption Plaques After Variable Periods of Incubation Under Control-MM With or Without Prior Incubation Under Antiserum-MM.[a]

Preincubation under antiserum-MM (days)	Days of incubation under control-MM			
	2	3	4	5
None	nd[b]	41	75	104
3	57[c]	65	82	nd
4	39	59	88	nd
5	50	75	92[d]	nd

[a] Numbers of plaques in duplicate flasks from each of two virus dilutions were summed (18).
[b] nd = not done.
[c] Total incubation time 5 days.
[d] Total incubation time 9 days.

specific immune serum blocks mumps virus hemadsorption (9, 17), so that even if plaques had developed, they could not have been visualized by the hemadsorption method. Since the strain of mumps virus used in these experiments does not produce cytolytic plaques, we used two indirect methods of assessing mumps virus spread in the presence of antiserum.

Firstly, flasks infected with a known number of plaque forming units (PFU) were incubated for 3–7 days under antiserum-MM, and then were rinsed and incubated a further 2, 3, or 4 days under control-MM before they were hemadsorbed. Addition of antiserum-MM was delayed until 6 hr after viral input to allow completion of viral penetration. From the size and number of hemadsorption plaques in these experimental flasks, compared with flasks incubated under control-MM for 3, 4, or 5 days before hemadsorption, conclusions could be drawn regarding the progression of infection under antiserum (see "Results" for further clarification). The size of a plaque was recorded as its greatest diameter and the diameter lying perpendicular to this, using a calibrated ocular micrometer. Mean plaque diameters were then calculated. Plaque areas were not used, because the variable shape of plaques made such calculations difficult.

Secondly, plaques were detected by immune cytolysis. After infection with 50–100 PFU of mumps virus, flasks were incubated for 4 or 5 days under antiserum- or control-MM. Representative flasks were then hemadsorbed, and the remainder were incubated overnight under either antiserum-MM or control-MM. The rabbit sera in the antiserum-MM and control-MM used for overnight incubation were heat-inactivated for some flasks, and not heat-inactivated for others, to test for complement-mediated immune cytolysis. Uninfected HeLa cell flasks were also incubated overnight with similar media, to serve as a control for possible anti-HeLa cell antibodies in the sera. After overnight incubation, the medium was removed, and the monolayers were stained with 0.5% aqueous trypan blue for 10 min, rinsed with BSS, and examined microscopically.

Results. Effect of mumps virus antiserum on evolution of hemadsorption plaques. Flasks incubated under control-MM and hemadsorbed at 3, 4, or 5 days after mumps virus infection showed an increase in number and size of hemadsorption plaques with time after infection, as previously described (14). Representative experimental flasks incubated for 3–7 days under antiserum-MM showed no hemadsorption, nor was virus present in the medium, thus confirming that the amount of antiserum had been adequate in suppressing release of infectious virus. However, hemadsorption plaques did develop in these experimental flasks after replacement of the antiserum-MM with control-MM.

In the experimental series of flasks pretreated for 3, 4, or 5 days with antiserum-

MM, then incubated under control-MM for a further 2, 3, or 4 days before hemadsorption, the number and size of plaques increased between the second and fourth day of the control-MM treatment. The results from this experiment, in Table I, demonstrate that the number of plaques in these experimental flasks depended not on the duration of the antiserum pretreatment but on the length of the control-MM treatment. If cell-to-cell spread of virus had continued during the antiserum pretreatment, the number of plaques in experimental flasks would have been expected to increase according to the total incubation time, regardless of whether antiserum was in the medium or not. This clearly was not the case, and the results show that mumps virus antiserum in the medium severely restricts cell-to-cell spread of the virus.

A uniform acceleration in plaque development, by about 1 day, did occur in all flasks receiving antiserum pretreatment, regardless of its length, compared with those control flasks incubated for a period of time equal to that of the control-MM treatment in experimental flasks (Table I). This probably reflects the amount of viral replication (to the stage of viral budding) which would continue in the initially infected cells even in the presence of antiserum.

Another experiment was designed to test the persistence of infection under antiserum. Flasks were incubated for 3, 5, or 7 days after infection under antiserum-MM and then for a further 3 days under control-MM before hemadsorption. Control flasks were incubated for 3, 4, or 5 days under control-MM before hemadsorption, as in the previous experiment. In flasks preincubated for 3 or 5 days under antiserum-MM before the 3 days under control-MM, the number of plaques showed slight acceleration, compared with the 3-day control flasks, as in the previous experiment. However, in flasks preincubated for 7 days under antiserum-MM, there was a dramatic reduction in numbers of plaques to about 4% of the 3-day control value. This suggests that few of the initially infected cells survived through 7 days of antiserum treatment, and were then capable of producing infectious virus on removal of the antiserum.

Although numbers of hemadsorption plaques developing in antiserum-treated flasks depended primarily on the duration of subsequent control-MM treatment, their size did increase slightly corresponding with longer incubation time under antiserum-MM. We interpret this to be due to possible accumulation of viral precursors during the 3–5 days of antiserum treatment, so that, on removal of the antiserum-MM, more progeny virions would be released, allowing a larger plaque to develop.

Effect of mumps virus antiserum on viral spread estimated by immune cytolysis. Flasks incubated under control-MM for 4–5 days after infection developed cytolytic plaques staining with trypan blue, after overnight exposure to antiserum-MM containing complement (Fig. 1a). These distinct foci of staining stood out against a background of diffuse single cell staining, and were similar in size and number to hemadsorption plaques in parallel flasks (Fig. 1b). In contrast, flasks treated with regular antiserum-MM for 4–5 days after infection, then treated overnight with antiserum-MM containing complement, lacked these cytolytic plaques (Fig. 1c), although diffuse single cell staining was present. These results show that spread of mumps virus infection to form plaques detectable by immune cytolysis had been prevented by antiserum, and thus confirm conclusions drawn from experiments testing viral spread by development of hemadsorption plaques.

The cytolytic plaques seen in infected flasks exposed overnight to mumps antiserum with complement were absent from similar flasks treated overnight with antiserum which had been heat-inactivated. This suggests that the plaques resulted from complement-mediated cell lysis of mumps virus infected cells in the presence of antiviral antibodies, as has been described for rabies and SV_5 infected cells (19, 20).

The diffuse single cell staining seen in all monolayers exposed to hyperimmune mumps antiserum which had not been heat-inactivated was most likely due to the presence in the antiserum of anti-HeLa cell

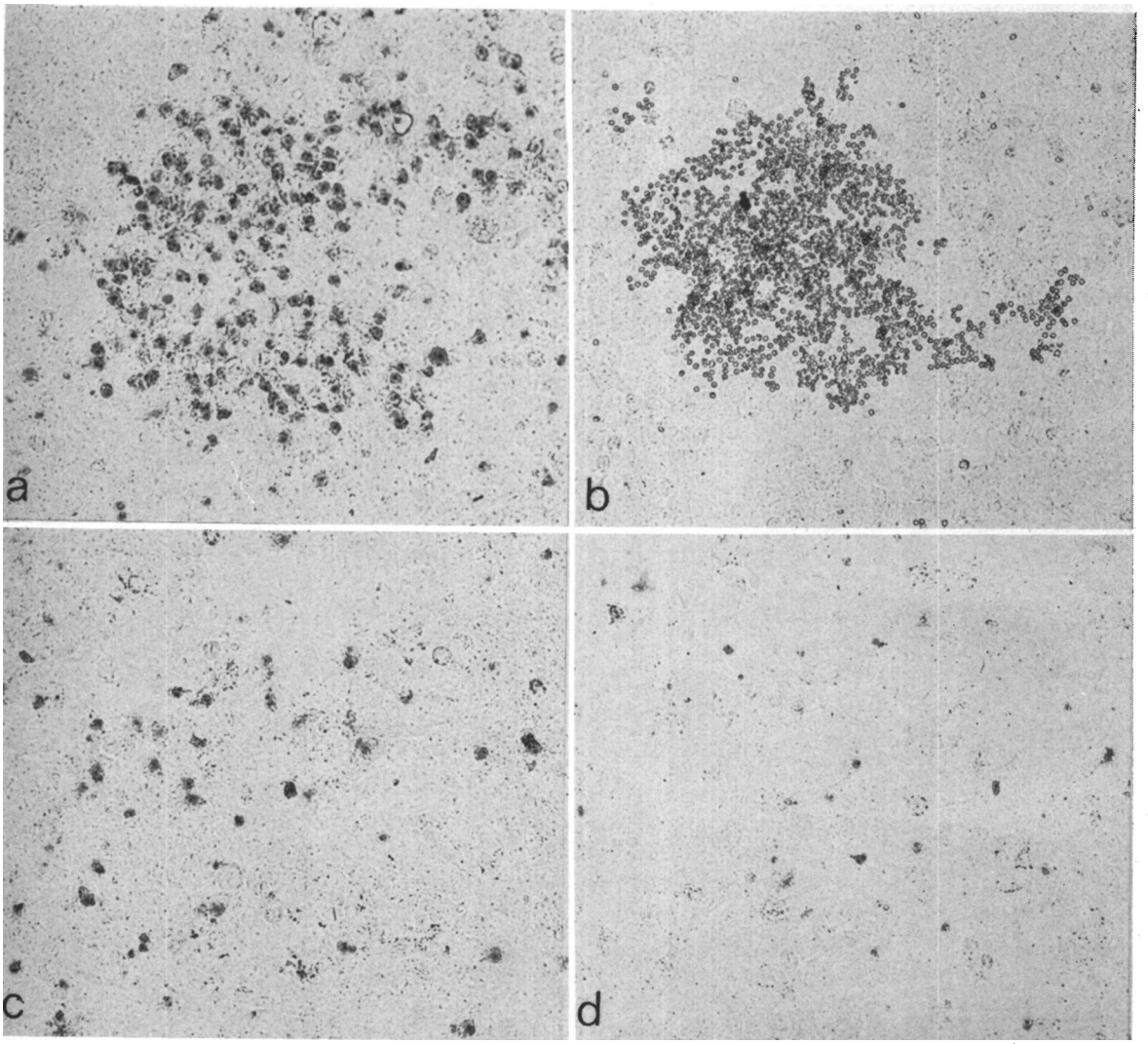

FIG. 1. Inhibition of development of mumps virus immune cytolytic plaques by antiserum: (a) HeLa cell monolayer incubated for 4 days after infection under control-MM, before overnight treatment with antiserum-MM, antiserum not heat-inactivated. Note trypan blue-staining immune cytolytic plaque, and background of diffuse single cell staining. (b) Hemadsorption plaque in parallel flask treated for 4 days after infection with control-MM. (c) Flask treated for 4 days after infection with antiserum-MM before overnight incubation under antiserum-MM, antiserum not heat-inactivated. No cytolytic plaques were present, but note diffuse single cell staining as in (a). (d) Flask treated as in (c), except that antiserum for overnight incubation had been heat-inactivated. Single cell staining reduced as compared with (c).

antibodies and complement, since a HeLa cell-propagated virus was used for hyperimmunization. This diffuse staining was also present in monolayers treated with uninactivated serum from rabbits inoculated with cell-free medium from uninfected HeLa cell cultures, but not in monolayers treated with preinoculation control rabbit serum containing complement. In all cases, the staining was considerably reduced in monolayers treated with the corresponding serum which had been heat-inactivated (Fig. 1d), as would be expected for a complement-dependent process.

Discussion. The present results show that

cell-to-cell spread of mumps virus is inhibited in the presence of specific immune serum, as is true for Newcastle disease virus and poliovirus (1, 2). In the system studied here, only a small proportion of mumps virus-infected cells went on to produce plaques when the antiserum-MM was left on for 7 days. This contrasts with results for Newcastle disease virus, parainfluenza 3 virus, poliovirus, and measles virus, where infected cells went on to produce progeny virus after long periods under the corresponding viral antiserum (2, 21–23). Treatment of a variety of rubella virus-infected cells with specific antiserum did not reduce numbers of infected cells (12, 13, 24) except in the case of infected Vero cells (24).

Cells infected with mumps (9), Newcastle disease (10), measles (11), and rubella (12, 13) viruses are capable of dividing, and this could serve as a means of viral spread or persistence in a culture treated with immune serum. However, antibody insensitive spread of progeny virus from an infected to an uninfected cell does not appear to occur in paramyxoviruses, with the exception of measles virus (8), and this virus is considered by many to be an atypical paramyxovirus (25). Viral spread in the presence of antibody, as occurs with herpesvirus (2, 6, 7), vaccinia virus (3–5), and measles virus (8) appears to relate to the ability of these viruses to cause cell fusion. Both mumps and Newcastle disease viruses are also capable of causing cell fusion as a result of infection. Interestingly enough, "fusion from within," the type of fusion which would result in spread of progeny virus, is inhibited by specific immune serum (26). The lack of direct cell-to-cell spread by these viruses in the presence of antiserum may therefore be secondary to inhibition of late viral induced cell fusion. The fact that spread of measles virus continues in the presence of antiserum suggests that the mechanism of fusion induction by measles virus may differ from that of other paramyxoviruses.

The present results *in vitro* support our findings in monkey embryos experimentally infected with mumps virus, where disappearance of infectious virus was paralleled by the appearance of maternal 7S neutralizing antibody (15). In the chick embryo, there appears to be a lag between disappearance of virus and the appearance of detectable amounts of neutralizing antibody (16), but further experiments are required to clarify this point. We have no indication that mumps virus persists intracellularly for any length of time after the appearance of antibody in either of these models, and results with mumps virus-infected HeLa cells suggest that such persistence would be unlikely. Persistence and spread, in the presence of antibody, of natural herpesvirus infections and of intrauterine rubella virus infection also find correlates in corresponding studies of viral persistence and spread *in vitro* in the presence of antibody, and emphasize the value of such an approach in understanding microepidemiological aspects of viral disease.

Summary. Cell-to-cell spread of mumps virus infection to form plaques in HeLa cell monolayers was studied in the presence and absence of mumps virus hyperimmune antiserum in the overlay medium. Using two techniques of plaque detection, viral spread was found to be inhibited by the antiserum. When the antiserum was removed after 3–5 days, plaque formation resumed, but after 7 days of treatment very few plaques developed, indicating limited persistence of mumps virus in HeLa cells in the presence of external neutralizing antibody.

1. Wheelock, E. F., and Tamm, I., J. Exp. Med. **113**, 301 (1961).

2. Black, F. L., and Melnick, J. L., J. Immunol. **74**, 236 (1955).

3. Nishmi, M., and Keller, R., Virology **16**, 91 (1962).

4. Nishmi, M., and Keller, R., Virology **18**, 109 (1962).

5. Postlethwaite, R., Virology **10**, 466 (1960).

6. Stoker, M. G. P., Nature (London) **182**, 1525 (1958).

7. Wheeler, C. E., and Canby, C. M., Arch. Dermatol. **79**, 86 (1959).

8. Rapp, F., Gordon, I., and Baker, R. F., J. Biophys. Biochem. Cytol. **7**, 43 (1960).

9. Walker, D. L., and Hinze, H. C., J. Exp. Med. **116**, 751 (1962).

10. Wheelock, E. F., and Tamm, I., J. Exp. Med. **113**, 317 (1961).

11. Rustigian, R., J. Bacteriol. **92**, 1792 (1966).

12. Maassab, H. F., and Veronelli, J. A., J. Bacteriol. **91**, 436 (1966).

13. Rawls, W. E., and Melnick, J. L., J. Exp. Med. **123**, 795 (1966).

14. Davis, C. W. C., and St. Geme, J. W., Jr., Proc. Soc. Exp. Biol. Med. **136**, 1319 (1971).

15. St. Geme, J. W., Jr., Davis, C. W. C., and Van Pelt, L. F., Pediat. Res. **5**, 379 (1971).

16. St. Geme, J. W., Jr., Davis, C. W. C., Cooper, M. D., and Noren, G. R., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **29**, 284 (1970).

17. Deinhardt, F., Bergs, V. V., Henle, G., and Henle, W., J. Exp. Med. **108**, 573 (1958).

18. Lorenz, R. J., Arch. Gesamte Virusforsch. **12**, 108 (1962).

19. Holmes, K. V., Klenk, H. D., and Choppin, P. W., Proc. Soc. Exp. Biol. Med. **131**, 651 (1969).

20. Wiktor, T. J., Kuwert, E., and Koprowski, H., J. Immunol. **101**, 1271 (1968).

21. Ackermann, W. W., and Kurtz, H., J. Exp. Med. **102**, 555 (1955).

22. Henle, G., Deinhardt, F., Bergs, V. V., and Henle, W., J. Exp. Med. **108**, 537 (1958).

23. Rustigian, R., J. Bacteriol. **92**, 1805 (1966).

24. Mifune, K., Desmyter, J., and Rawls, W. E., Infec. Immunity **2**, 132 (1970).

25. Scholtissek, C., Drzeniek, R., and Rott, R., *in* "The Biochemistry of Viruses" (H. B. Levy, ed.), p. 219. Dekker, New York (1969).

26. Bratt, M. A., and Gallaher, W. R., Proc. Nat. Acad. Sci. U.S.A. **64**, 536 (1969).

Received Sept. 13, 1971. P.S.E.B.M., 1972, Vol. 139.