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Plaque Formation by Mumps Virus and Inhibition by Antiserum

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Boston and ABC strains of mumps virus produced plaques approximately 1.0 mm in diameter in monolayers of BGM cells. The plaques were circular and either clear or target-like in form. Ricki strain virus produced plaques of similar size and form but, in addition, a red plaque was observed with this agent. The vaccine strain of mumps virus, Jeryl Lynn, produced minute clear plaques approximately 0.3 mm in diameter. Incorporation of diethylaminoethyl (DEAE)-dextran into the overlay medium did not affect the size difference between Jeryl Lynn plaques and those of the other strains. However plaques of the Jeryl Lynn and Ricki strains were more easily visualized when the overlay medium contained 400 μg/ml of DEAE-dextran. Simultaneous titration by plaque formation and roller tube infectivity showed that these two methods were of equal sensitivity. Virus neutralization by antibody was demonstrated by plaque reduction. Rise in antibody titer was observed in sera from human and animal infection, human vaccination, and rabbit immunization.

The introduction of live virus mumps vaccine has stimulated virological studies on various mumps strains. Plaque characteristics have been very useful in studies of differences of virus strains and their attenuation. In addition, neutralization tests by plaque reduction also have provided an important means of measuring antibody response and investigating neutralization kinetics.

Frothingham and Granoff (5) first described mumps plaques in monolayers of chick embryo fibroblasts and HeLa cell. Gresser and Enders (6) later reported plaque formation by chick embryo-adapted virus in chick embryo fibroblast cell cultures and failure of this strain to produce plaques in human amnion cultures. They showed that a mumps virus strain adapted to growth in the latter cell culture system would produce plaques. Hotchin, Deibel, and Benson (7) developed a procedure in which foci of mumps virus growth were located by the hemadsorption technique. Recently, Ennis and co-workers (3) have described a plaque neutralization test for the detection of mumps antibody.

This report documents characteristics of plaque formation by various mumps virus strains as well as a plaque reduction neutralization procedure for assay of mumps antibody. The system used has been previously reported (1, 4).

MATERIALS AND METHODS

Cell cultures. A continuous line of African green monkey kidney cells, designated BGM, was used. The growth medium consisted of Eagle's minimal essential medium (MEM) in a base of Earle's balanced salt solution, 10% newborn calf serum, and 50 μg per ml of Aureomycin (chloretracycline; Squibb). Cells were implanted in 30-ml plastic flasks (Falcon Plastics) or in glass culture tubes (16 by 125 mm) at a concentration of 80,000 to 90,000 cells per ml. Monolayers were usually complete on the 5th day, and the cultures were ready for use. In virus experiments employing tube cultures, Eagle's basal essential medium in Earle's salt solution, 3% newborn calf serum, and 50 μg per ml of chlortetracyline was used as maintenance medium.

Virus. The virus strain which was used in most experiments was an agent obtained from I. Gresser, Paris, France. We have referred to this agent previously as the Boston strain (4). The virus was isolated in primary human amnion cell culture and was passaged 10 times in these cells. Pools of virus for use in this investigation were prepared as 1st to 3rd passage in BGM cells. The live virus vaccine strain, Jeryl Lynn, was obtained from P. Isacson, Buffalo, N.Y. The agent was supplied as lyophilized chick embryo tissue culture material and was reconstituted with sterile distilled water before use. ABC strain was obtained from M. R. Hilleman, West Point, Pa., as monkey kidney cell culture material. The agent had a history of seven passages in HeLa cells and at least four passages in monkey kidney cell culture. The pool studied in this investigation was prepared in rhesus monkey kidney cells. The Ricki strain was received from W. Henle, Philadelphia, Pa., as allantoic fluid. The preparation employed in this investigation was 3rd chick embryo passage allantoic fluid. The Enders

strain was obtained from D. T. Karzon, Nashville, Tenn., as allantoic fluid.

Plaque technique. Flasks were emptied of growth medium, and 0.5 ml of virus preparation was added. Virus dilutions were made in Temin's modification of Eagles' MEM (Grand Island Biological Co., Grand Island, N.Y.). The flasks were incubated at 36 C for 2 hr to allow adsorption of virus. Monolayers were overlayed with 3.5 ml of medium consisting of Temin's medium, 1% calf serum, and 1.5% Noble agar (Difco). Flasks were inverted and incubated at 36 C. Staining of monolayers was carried out by the addition of a second overlay of plaquing medium which contained 0.01% neutral red stain.

In some experiments DEAE-dextran (Pharmacia, Uppsala) was incorporated into the overlay medium in various concentrations.

Plaque measurement. In most experiments measurement of plaque size was carried out with a millimeter scale and estimated to the nearest 0.1 mm. In some experiments, more precise measurement of plaque size was made by using a Nikon model 60 Shadowgraph. Diameters were measured to 0.001 mm, and averages and standard deviations were computed from these data.

Virus titrations. Infectivity titrations were carried out in the following manner in roller tube cultures. Five tubes of BGM cell cultures were inoculated with 0.1 ml from each virus dilution and incubated on a roller drum at 36 C. Cultures were observed daily for the development of cytopathic effect (CPE) for 6 days subsequent to inoculation. Hemadsorption (HAD) tests using 1% guinea pig erythrocyte suspension were performed on cultures not showing CPE after 6 days of incubation. End points of infectivity titrations were taken as the highest dilution showing positive for CPE or HAD, or for both; 50% tissue culture infectious doses ($TCID_{50}$) were calculated by the method described by Hsiung (8).

Plaque titrations were carried out by inoculation of serial 10-fold dilutions of virus into each of two flasks. Flasks were incubated for 5 days at which time the second overlay was added. The plaques were counted on day 6, except for those of the Ricki strain which were counted on day 7. Infectivity was expressed as plaque-forming units (PFU) per ml. This protocol was established on results to be described.

Antisera. Rabbit antiserum was prepared against purified V antigen from Enders strain. The antigen was prepared by a method modified slightly from that described by Ogiwara and Matumoto (9). The modification consisted of using 0.1% Tween 80 in addition to ether in the disruption of virus. Also, absorption and elution of hemagglutinin was carried out at *p*H 6.0 rather than *p*H 7.0. The V antigen was incorporated into an equal volume of Freund's complete adjuvant (Difco) and inoculated intradermally. An additional 1 ml of V antigen alone was administered intravenously. The serum was collected 6 weeks after injection.

Serum samples were obtained from rhesus monkeys which were infected with mumps virus. Infection was carried out by injection of the Boston strain into the parotid gland by way of Stenson's duct.

Human sera from patients with mumps virus infection (parotitis, orchitis, meningoencephalitis) were obtained from E. H. Lenette, Berkeley, Calif., and the Erie County Virology Laboratory, Buffalo, N.Y. Sera were also obtained from persons that had received live-virus vaccine. These sera were donated by P. Isacson.

Plaque reduction test. Neutralization of mumps virus infectivity by antisera was measured by a plaque-reduction test. Fourfold dilutions of heat-inactivated antisera (56 C, 30 min) were added to equal volumes of Boston strain virus in suitable dilution, containing approximately 200 PFU/ml. Virus-serum mixtures were incubated for 1 hr at room temperature, after which 0.5 ml of each mixture was inoculated into each of two flasks. The rest of the test was identical to that described above for plaque technique. Exact virus input was determined in each experiment from plaque counts of flasks inoculated with mixtures of virus and normal serum.

Titers were derived by plotting per cent input against serum dilution and determining the 50 and 80% reduction titers from the curve.

RESULTS

Plaque characteristics of Boston and other strains. Experiments were performed in which a series of flasks was inoculated with an appropriate dilution of Boston strain virus. Each day after inoculation, two flasks were stained, returned to incubation, and observed for plaques

TABLE 1. *Number of Boston strain mumps virus plaques in relation to day after inoculation*

Day stained	Day plaques were counted							
	2	3	4	5	6	7	8	9
1	10[a]	21	30	Faded				
2		23	31	Faded				
3			48	64	Faded			
4				77	73	Faded		
5					75	75	66	Faded
6						76	76	Faded
7							77	72
8								77

[a] Total number of plaques in two flasks.

after 1 day. Table 1 shows the plaque counts for a typical experiment of this type. As shown in the table, the apparent number of plaques increased on day 2 through day 5 after inoculation. After day 5 the number did not increase to any significant degree. Since plaque counts did not increase after day 5, titration protocols were established for stain on day 5 and count on day 6.

In these experiments, flasks were retained in incubation after staining and examined on each subsequent day for changes in plaque number and plaque morphology. It is interesting to note that plaque counts on day 4 of flasks stained on day 1 and day 2 were lower than those of flasks stained on day 3. The observation and accurate counting of plaques became difficult after 2 to 3 days of stain.

During this experiment, estimates of plaque size

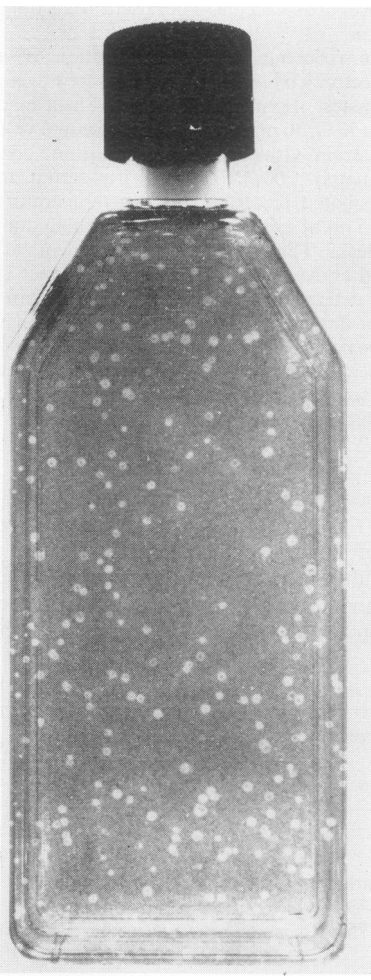

FIG. 1. *Boston strain plaques in a monolayer of BGM cells 6th day after inoculation.*

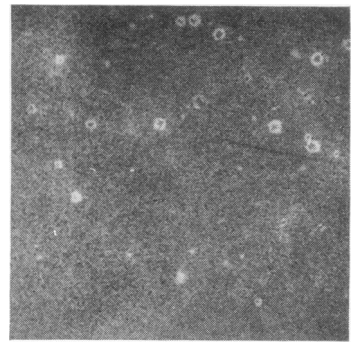

FIG. 2. *Target-like plaques produced by Boston strain in BGM cell cultures.*

were made on plaques appearing 24 hr after staining. Plaques on day 2 were less than 0.5 mm in diameter. On day 4, 60% of plaques ranged between 0.8 and 1.0 mm, the remainder were smaller. On days 5 through 8, when maximum size was obtained, 80% of plaques were estimated to be 1.0 to 1.2 mm in diameter. Boston strain plaques on day 2 and day 3 were clear, circular unstained areas in the monolayers. Most plaques observed on subsequent days were also clear and circular. In addition a second type of plaque was observed, on approximately day 4. This plaque was target-like in appearance. It consisted of a central stained area surrounded by a peripheral clear zone. Target-like plaques on day 6 were approximately 30% of the population, whereas on day 7 there were fewer such plaques, even though the total number of plaques remained unchanged. This suggested that the target-like plaques changed to clear forms. Figure 1 shows the appearance of Boston strain plaques on day 6. Figure 2 shows the target-like plaques. Beyond day 7, in some experiments, streaking or tailing was observed.

ABC strain virus produced plaques which first were observed on day 2. The number increased to maximum level on day 4 and remained constant thereafter through day 9; 90% of ABC plaques on day 2 were clear areas, less than 0.5 mm in diameter. Plaques on day 3 were somewhat larger, 30% being approximately 1.0 mm in diameter and the remainder smaller. Plaques on days 4, 5, 6, and 7 were large, 1.0 mm or greater in diameter. Target-like plaques were seen in monolayers after day 3, but never exceeded 10% of the population (Fig. 3).

Ricki strain plaques were first observed on day 2 after inoculation. Counts were difficult to obtain at this time because of the lack of definition of the plaques. Accurate counts could not be made before day 7. Plaques on day 2 were faint and non-

distinct. Many areas were observed in which the neutral red dye appeared concentrated, forming a "red" plaque. Day 3 observations showed faint, nondistinct plaques, which nonetheless were of the clear type, as well as "red" plaques and target-like forms. On days 4, 5, and 6, all three forms were observed. Plaques on day 7 were mostly clear or target-like in form. Target-like plaques formed 65% of the population at this time (Fig. 4).

Low-power microscopic examination showed that target-like and clear plaques of Boston, ABC, and Ricki strains were syncytial in nature and that nuclei were aggregated in the center of the plaque. Plaques showing the target-like appearance had the neutral red stain concentrated about the nuclei in the syncytium. Red plaques did not appear to be syncytial.

The Enders strain did not produce plaques in BGM monolayers when inoculated in dilutions ranging from undiluted to 10^{-6}. We were unable to detect replication of this agent through five passages in tube cultures of BGM cells when examined for CPE, HAD, or development of hemagglutinin when back-passaged in 8-day chick embryos.

Characteristics of Jeryl Lynn strain plaques. Similar experiments to those described above were

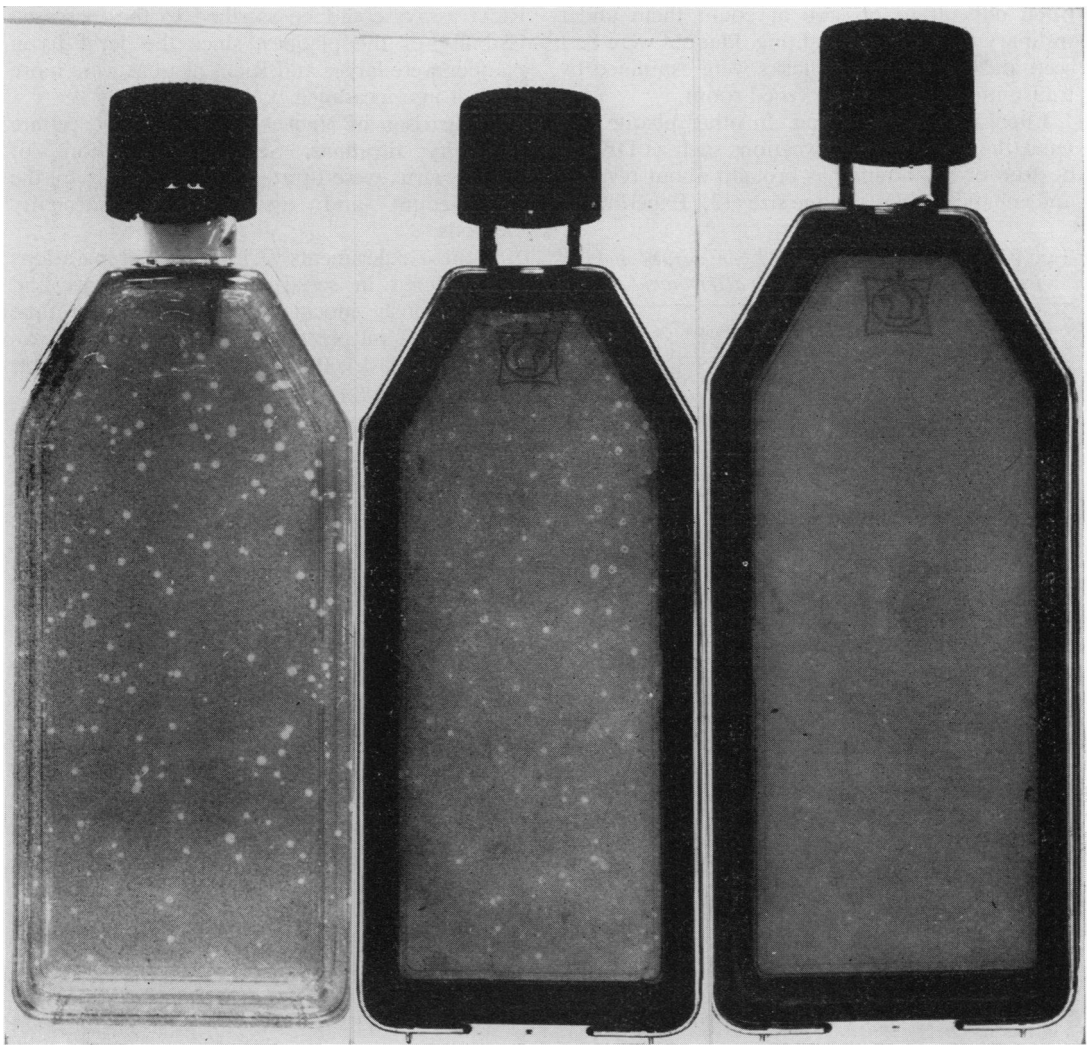

FIG. 3. *ABC plaques in a monolayer of BGM cells 6th day after inoculation.*
FIG. 4. *Ricki plaques in a monolayer of BGM cells 6th day after inoculation.*
FIG. 5. *Jeryl Lynn plaques in a monolayer of BGM cells 6th day after inoculation.*

carried out with the Jeryl Lynn agent. Table 2 shows the results of an experiment in which Jeryl Lynn strain was observed for time of plaque appearance and count.

Jeryl Lynn plaques were first detected on day 3. The number increased on day 4 and remained constant through day 7. The plaques were minute clear areas, approximately 0.3 mm in diameter (Fig. 5). No detectable change in size or form was noted throughout this or other experiments. Red plaques or target-like plaques have not been observed in Jeryl Lynn-infected monolayers.

Microscopic examination of Jeryl Lynn plaques showed the plaques were also syncytial in nature. Because of the small size of these plaques, it was often difficult to observe or count them under ordinary conditions of lighting. Plaques were best seen and counted when flasks were examined by transmitted light in a darkened room.

Effect of DEAE-dextran. In other plaque systems, the addition of polycations such as DEAE-dextran or protamine has brought about reversal and enhancement of plaque size (13). Experiments were performed to determine the effect of DEAE-dextran on mumps virus plaques in BGM cells. Suitable dilutions of Boston, ABC, Ricki, and Jeryl Lynn strain viruses were inoculated into flasks and overlaid with medium containing various concentrations of DEAE-dextran. Monolayers were stained on day 5 and counted on day 6. Table 3 shows the number of plaques counted, average diameters, and standard deviations of plaque diameters in these experiments. There was a slight increase in the size of the plaques of the Boston and Ricki strains. The size of Jeryl Lynn plaques increased to a greater degree yet did not approach the size found with the other strains. The increase in number seen in the Jeryl Lynn and Ricki assays could be ascribed to the increased visibility of the plaques, since the Jeryl Lynn plaques were larger and Ricki plaques were more distinct in appearance.

TABLE 2. *Number of Jeryl Lynn strain mumps virus plaques in relation to day after inoculation*

Day stained	Day plaques were counted					
	2	3	4	5	6	7
1	0	10[a]	Faded			
2		12	27	Faded		
3			88	61	Faded	
4				87	88	Faded
5					89	86
6						87

[a] Total number plaques in two flasks.

Comparison of plaque-count and tissue culture infectivity titrations. Several preparations of mumps virus were titrated simultaneously by the plaque-count and tissue culture infectivity methods.

In these experiments various virus preparations were diluted in serial 10-fold increments and simultaneously inoculated into five roller tube cultures (0.1 ml per tube) and two plaque flasks (0.5 ml per flask). Plaque assays were carried out with 400 μg of DEAE-dextran added to the overlay medium. Table 4 shows the results of these experiments. Results indicated essentially equal sensitivity of these titration methods. The Jeryl Lynn strain was notably lower in titer than the other three strains by both methods. Infectivity titers of Jeryl Lynn strain obtained in this study were similar to those reported by Buynak and

TABLE 3. *Effect of DEAE-dextran on mumps virus plaques*

Mumps strain	Concn of DEAE-dextran								
	None			400 μg per ml			800 μg per ml		
	No.[a]	Size[b]	SD[b]	No.	Size	SD	No.	Size	SD
Boston	153	0.85	0.17	149	0.95	0.23			
ABC	464	0.98	0.20	482	0.99	0.22			
Ricki									
Expt 1	51	0.87	0.21	149	0.95	0.28			
Expt 2	29	0.80[c]	0.18[c]	83	0.85	0.16	80	0.82	0.16
Jeryl Lynn									
Expt 1	58	0.31	0.10	176	0.46	0.12			
Expt 2	119	0.33	0.10	171	0.45	0.13	162	0.48	0.21

[a] Total number of plaques in two flasks.

[b] Average diameters are expressed in millimeters, and standard deviation values are computed from measurement of 50 plaques.

[c] Computed from measurement of 25 plaques.

Hilleman (2). These authors reported titers obtained in primary grivet monkey kidney cells.

Measurement of mumps-neutralizing antibody. Neutralization of mumps virus infectivity by antibody was detected and quantitated by a plaque reduction test in BGM monolayers. Tests were carried out with rabbit sera, monkey sera, and human sera secured from clinical cases and from vaccinees. Figure 6 shows characteristic curves for neutralization tests of various sera against Boston strain virus. These curves relate the neutralizing capacity of antiserum dilutions to input infectivity. The data reflect the response in neutralizing antibody titers to natural and experimental infection and immunization with replicating and nonreplicating antigens.

The effect of DEAE-dextran in the overlay on plaque reduction by neutralizing antibodies was studied. A comparison of plaque reduction titers of a single serum, obtained in three experiments,

TABLE 4. *Comparison of plaque titer to $TCID_{50}$*[a]

Strain	PFU/ml	$TCID_{50}$/ml
Boston	1.9×10^6	$10^{5.6}$
ABC	6.3×10^7	$10^{7.6}$
Ricki	9.3×10^6	$10^{7.2}$
Jeryl Lynn	2.4×10^4	$10^{4.2}$

[a] $TCID_{50}$ = median tissue culture infective dose.

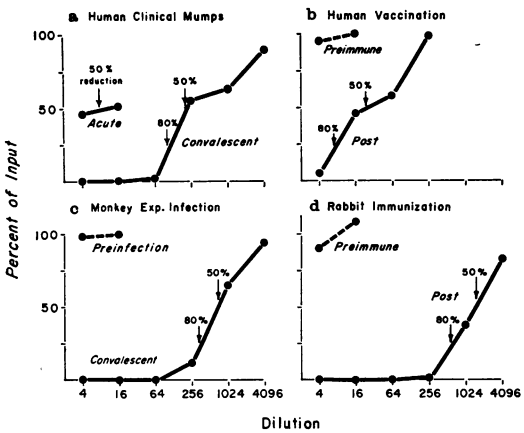

FIG. 6. *(a) Plaque reduction by acute and convalescent phase sera from a human case of mumps. (b) Plaque reduction by pre- and postvaccination sera from a child that received live virus (Jeryl Lynn) vaccine. (c) Plaque reduction by pre- and postinfection sera from a monkey experimentally infected with Boston strain of mumps virus. (d) Plaque reduction by pre- and postimmunization sera from a rabbit immunized with purified mumps V antigen.*

TABLE 5. *Influence of DEAE-dextran in overlay on results of plaque reduction tests*

Expt[a]	Concn of DEAE-dextran			
	None		400 μg/ml	
	50% Reduction	80% Reduction	50% Reduction	80% Reduction
1	2,450	407	708	309
2	596	309	708	309
3	1,740	407	1,410	407

[a] Three plaque reduction tests with the same rabbit antiserum run against Boston strain of mumps virus.

TABLE 6. *Detection of neutralizing antibody in serum from an experimentally infected monkey*

Day after infection	Plaque reduction titer	
	50%	80%
0	<4	<4
10	234	68
21	512	219
35	617	269

is shown in Table 5. The data suggest that incorporation of DEAE-dextran in the overlay tended to reduce variations in observed titers when the 50% reduction end point was used. No effect was noted when 80% reduction end points were used. Since DEAE-dextran improved the definition of plaques and did not affect the neutralization test, it was routinely used at a concentration of 400 μg/ml in overlay medium.

Table 6 shows plaque reduction titers of serial bleedings from a monkey infected with mumps virus. The data reflect the response in neutralizing antibody titers in an experimentally infected animal. A significant titer of antibody was detected on the 10th day after infection. Antibody levels continued to rise through the 35th day. The magnitude of titers indicates a high degree of sensitivity for the detection of antibody.

DISCUSSION

Four morphological forms of mumps virus plaques were observed in this study. Two of these forms, the target-like plaque and the large clear plaque were seen in monolayers infected with Boston and ABC strains. These forms were also observed among Ricki strain plaques which had in addition red plaques. The fourth form was a minute clear plaque which was observed only with the Jeryl Lynn strain. Comparison of the proportion of target-like forms to clear forms on

successive days suggested a change from target-like to clear forms. Schloer and Hanson (11) described Newcastle disease virus (NDV) plaques in chick embryo fibroblast cultures which evolved from a red plaque through a target-like form to a clear plaque. Although this evolution is compatible with our data, this does not mean that all of the clear plaques follow this process of formation. Our earliest observations of Boston, ABC, and Ricki plaques were of the clear form. Plaque size of Boston, ABC, and Ricki strains increased from day 2 through day 5, paralleling somewhat the increase in plaque number during this period. Largest plaques observed were approximately 1.2 mm in diameter.

Jeryl Lynn plaques were distinctly different from the other strains. Plaque size was approximately 0.3 mm throughout incubation periods of 7 days. The plaques were more uniform in size and form than were the plaques produced by other mumps virus strains.

Attenuation in virulence and small plaque size have been associated by various authors. Schloer (Bacteriol. Proc., p. 122, 1964) demonstrated decreased virulence of NDV small plaque mutants for chick embryos and 1-day chicks. Rapp (10) documented small plaque formation by vaccine strains of measles virus as compared to large plaques formed by wild strains of measles virus. Our observation of the small plaque size of the Jeryl Lynn vaccine strain of mumps virus also associates small plaque size with attenuation of the virus strain.

The size difference of plaques between Jeryl Lynn strain and other strains of mumps virus was not altered by the addition of DEAE-dextran to the overlay medium. Although slight enhancement in plaque size was noted, the characteristic difference in size of plaques between strains was not affected by DEAE-dextran. The formation of small plaques by viruses has been suggested (12) to be either a result of an increased capacity to stimulate interferon, or a lower virus yield per infected cell, or a sensitivity to conditions in the medium which are alleviated by the incorporation of polycations. The data presented here tend to eliminate the last suggestion as the responsible mechanism for the small size of Jeryl Lynn plaques.

Plaque reduction tests demonstrated an increase in neutralizing antibody in a variety of situations. Antibody rises were detected in human sera secured from clinical cases of mumps virus infection or after vaccination with live virus vaccine, as well as in monkey sera obtained from experimentally infected animals. The tests also showed a response to nonreplicating viral antigen in the case of a rabbit immunized with V antigen.

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