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Reduction in Plaque Size and Reduction in Plaque Number as Differing Indices of Influenza Virus-Antibody Reactions

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ABSTRACT

JAHIEL, R. I. (Cornell University Medical College, New York, N.Y.), AND E. D. KILBOURNE. Reduction in plaque size and reduction in plaque number as differing indices of influenza virus-antibody reactions. J. Bacteriol. **92**:1521–1534. 1966.—The serological reactivity of an antigenically hybrid influenza virus recombinant (X-7) was studied in a heteroploid cell plaquing system in which antisera to the parental viruses NWS/(A_0) and RI/5^+ (A_2) were incorporated in agar overlay media. Two different effects on plaque formation were found. With NWS antiserum, there was close relationship between reduction in plaque size and in plaque number [plaque inhibition (PI) pattern]. With RI/5 antiserum, plaque size reduction (PSR) occurred over a wide zone of serum dilutions without concomitant change in plaque number (PSR pattern). Several different preinoculation neutralization tests showed a strong reactivity of X-7 with NWS antisera and little if any reactivity with RI/5 antisera. We interpret the differing effects of NWS and RI/5 antisera on X-7 as indicative of the possible occurrence of different mechanisms of neutralization or of the possible participation of different surface antigens. The kinetics of PSR are consistent with the hypothesis that it results from the reaction of RI/5 antiserum with the RI/5-like neuraminidase of X-7. Studies with the antiserum-in-overlay technique of PSR and PI patterns comprise a sensitive method for quantitative antigenic analysis of plaque-forming influenza viruses.

When cell cultures inoculated with poliovirus are incubated under an overlay containing a homologous or a cross-reacting heterologous antiserum (19), two end points may be observed: decrease in number of plaques and decrease in size of plaques (19). The Wecker technique has been used extensively for the intratypic differentiation of polioviruses, with decreased plaque size (14, 18, 19) or decreased plaque number (12) as end points. Yet, there have been no reports describing the way in which the results obtained with these two end points vary with serial serum dilutions or with serial bleedings during immunization; thus, it is not known whether there are different patterns of relationship between reduction in plaque size and reduction in plaque number. Furthermore, it is not known whether there is a consistent relationship between the serum concentrations required to obtain decreased plaque size or decreased plaque number with the Wecker technique and those concentrations required to obtain decreased plaque numbers in the conventional (preinoculation) neutralization tests. The interest of this information extends beyond its applicability to the fine antigenic characterization of viruses. The demonstration of different patterns of plaque size reduction and plaque number reduction in the antiserum-in-overlay test and in conventional neutralization tests could point to different mechanisms of virus neutralization, or to basic differences in the antigenic structure of viruses.

During studies of recombinants of A_0/NWS and A_2/RI/5^+ influenza viruses (4, 6), a recombinant with dual antigenic reactivity was isolated (E. D. Kilbourne et al., Perspectives Virol., *in press*). This recombinant, X-7, was initially characterized in hemagglutination-inhibition tests as NWS-like, but with "minor" RI/5^+-like reactivity in strain-specific complement-fixation tests and in mouse infection. In addition, incorporation of NWS or RI/5^+ antisera into agar

overlays in a stable cell line plaquing system (16) resulted in complete plaque inhibition (PI) of X-7 with X-7 or NWS antisera. However, a different effect, plaque size reduction (PSR), resulted with antiserum to the RI/5$^+$ parent. These differing patterns of antigenic reactivity and their implications are the subject of the present report.

MATERIALS AND METHODS

Cells. Clone 1-5C-4, a human heteroploid cell derived from a variant line of the Chang conjunctival cell (21), was used. The methods for growth and maintenance of this clone have been described (16).

Viruses. X-1L and X-7 were the products of recombination between NWS-MK-p (an A_0 strain) and RI/5$^+$ (an A_2 strain). The parent strains, as well as the related strains NWS-34, RI/5$^-$,RI/5$^+$ (MK), and RI/5$^-$ (MK), and the influenza B/Lee strain have been described (5, 16). The experiment in which these recombinants were obtained is described elsewhere (E. D. Kilbourne et al., *in press*). Significant features of that experiment, as well as the further passage and cloning history of the virus, are summarized in Fig. 1. Recombinant X-7 (F1) was isolated after recombination of X-7 and RI/5$^+$ (MK).

Plaque assay techniques. The preparation of 60-mm Falcon dish cultures and the procedures for virus inoculation and adsorption have been already described (16). The agar overlay was Sugiura and Kilbourne's serumless medium 1 (16). Incubation was at 35 C in a humidified atmosphere of 3% CO_2, for 4 days, unless stated otherwise. The overlay was removed by gently separating it from the upturned plate with forceps. The plates were washed once with phosphate-buffered saline, and the cultures were fixed and stained in situ by immersion for about 1 min in 0.1% crystal violet in 20% ethyl alcohol in water, rinsing in tap water, and drying. Alternatively, monolayers were fixed in Bouin's solution, stained with Mayer's hematoxylin for 10 to 30 min, rinsed with 5% $NaHCO_3$ and distilled water, and then dried. The plaque size was the same with both techniques, but the Bouin-hematoxylin technique allowed better visualization of cellular details. With both techniques, the pyknotic, rounded cells within the plaque (those which do not stain when a neutral red-containing overlay is used) were washed off, leaving a space. The monolayers had a cell population density of 500 to 2,000 cells per square millimeter; the occasional occurrence of holes smaller than 0.01 mm^2 imposed a lower limit to the size of the plaques that could be detected.

Plaque counts. These were performed by scanning the entire plate with a microscope (Bausch & Lomb Tri-Simplex), projecting vertically the image of the plaque at a magnification of 21 times or higher.

Plaque size measurements. The outline of the projected image of the plaque was traced at a magnification of 21 times or higher. The points at the periphery of the plaque where the cell population density was half that of the monolayer represented the circumference of the plaque. These points were clearly defined in the case of X-7 plaques, which showed a very sharp fall in cell population density at the edge of the plaque. Because the plaques were very nearly circular, their size could be accurately measured by superimposing on the plaque tracing a transparent medium on which concentric circles with 2.1-cm radial increments were drawn. The plaque's radius and surface were fitted to those of the nearest circle. Comparison of the results obtained with this technique and those obtained with a polar planimeter on the same tracings showed good agreement and no

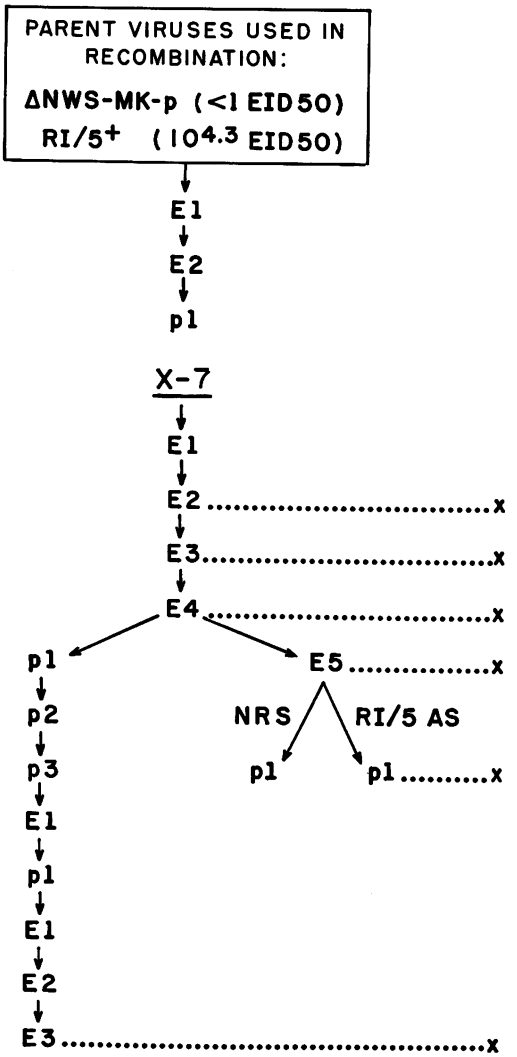

FIG. 1. *Origin, cloning, and passage history of X-7. Symbols: E, allantoic passage; En, consecutive allantoic passage after recombination (above X-7) or plaque isolation (below X-7); p, plaque isolation; pn, n^{th} consecutive plaque isolation; NRS, normal rabbit antiserum in overlay during plaque isolation; RI/5 AS, RI/5 antiserum in overlay during plaque isolation;* ×, *PSR demonstrated with RI/5 antiserum in overlay;* △, *heat inactivation at 40 C/96 hr.*

unidirectional error. Unless stated otherwise, the size of all the plaques in the plate was measured.

Antisera. Antisera were prepared in rabbits by initial intravenous injection of $10^{3.6}$ to $10^{5.0}$ hemagglutination (HA) units of allantoic fluid virus concentrated by ultracentrifugation, followed by intraperitoneal injections of $10^{4.7}$ to $10^{5.6}$ HA units at 7 to 10 days and finally $10^{5.3}$ to $10^{6.2}$ HA units at 15 to 20 days and at subsequent injections. An RI/5$^-$ antiserum produced by a different immunization procedure was obtained from W. G. Laver and R. Webster of the Australian National University in Canberra. To produce this serum (LW5), allantoic passage RI/5$^-$ virus was adsorbed to red blood cells and eluted and purified further by sucrose gradient centrifugation; two samples of the purified preparation, containing 3,000 HA units each, were injected intravenously, at 40-day intervals, and the rabbit's blood was collected 7 days after the last injection. All sera were heated for 30 min at 56 C prior to use, but were not otherwise treated.

Virus neutralization tests in ovo. Equal volumes of serial serum dilutions and of virus diluted to yield 10^2 EID_{50} per 0.1 ml were mixed and allowed to react at 4 C for 1 hr, and 0.1 ml of the mixture was inoculated into the allantoic cavity of 12-day-old chick embryos. After 40 hr of incubation at 37 C, the allantoic fluids were collected, diluted 1:4 with phosphate-buffered saline, and tested for hemagglutination with human O red blood cells as described previously (21).

"Postinoculation neutralization" (antiserum-in-overlay test or Wecker technique). Adsorption, addition of overlay, and inoculation of 20 to 150, or more, PFU (plaque-forming units) of virus were performed as in the usual plaque assay (16), except that the overlay contained dilutions of antiserum. The inoculum was not removed routinely, because comparative experiments had shown that this had little effect in this system on the number and size of plaques. The end points were the number and size of plaques after 4 days of incubation unless stated otherwise.

The abbreviation "PSR" is used to describe a plate in which the size of plaques is decreased as compared with controls. The abbreviation "PI" is used in this paper to describe a plate in which the number of plaques is decreased as compared with the control, or a plate in which there are no plaques. The abbreviations "PSR titer" (plaque size reduction titer) and "PI titer" (plaque inhibition titer) are used to refer to the point at which a 50% decrease in median plaque radius and number of plaques per plate, respectively, occurred. The abbreviation "PSR pattern" is used to refer to a titration in which plaque size reduction without plaque number reduction was present over a wide range of dilutions; the abbreviation "PI pattern" is used to refer to a titration in which plaque size reduction (PSR) occurred only in close association with reduction in plaque numbers (PI). The above abbreviations are used in the text only when it appears that their use is unambiguous. The above terms and their abbreviations are used only in reference to the antiserum-in-overly neutralization test.

Hemagglutination and hemadsorption tests. The hemagglutination (HA) and hemagglutination-inhibition (HI) titration methods have already been described (21). Microscopic hemadsorption (MHA) was performed by washing the plates twice with about 5 ml of phosphate-buffered saline after removing the agar overlay, and then adding 2 ml of a 0.1% (v/v) suspension of human group O red blood cells. The cells were allowed to react for 10 to 15 min at room temperature before they were washed twice gently with phosphate-buffered saline and fixed in Bouin's solution. Hemadsorption, which could sometimes be seen grossly, was determined microscopically, after fixation or after further staining in hematoxylin. Hemadsorption allowed the detection of plaques of radius smaller than 0.1 mm. A hemadsorption plaque is illustrated in Fig. 2.

RESULTS

Initial observation. Cultures inoculated with 60 PFU of X-7 were incubated under overlays containing NWS or RI/5 antiserum or normal rabbit serum in a final dilution of 1:400. After 4 days of incubation, there were no plaques in the plates with the NWS antiserum, whereas the plates with the RI/5$^+$ antiserum showed the same number of plaques as the control, but with markedly reduced plaque size. This result, which resembled the observations of Wecker (19) on

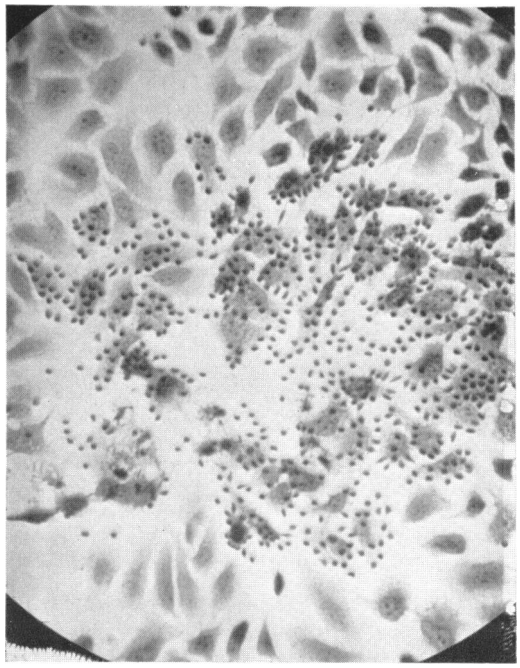

FIG. 2. *Plaque demonstrated by microscopic hemadsorption. X-7 plaque after 4 days of incubation under an overlay containing RI/5$^+$ antiserum. Hemadsorption following by Bouin fixation and hematoxylin staining.* × *230.*

poliovirus plaques under homologous to heterologous antisera, could be explained in two ways. (i) Both sera might exhibit, on serial dilution, sequential zones of plaque inhibition and plaque size reduction. In the case of the NWS antiserum, the single dilution of antiserum happened to fall in the plaque inhibition zone, and in the case of the RI/5$^+$ antiserum in the plaque size reduction zone. (ii) The NWS and RI/5$^+$ antisera, when incorporated in the overlay, show two intrinsically different patterns of neutralization of X-7, one of which is associated with plaque inhibition and the other only with plaque size reduction.

Effect of serial dilutions of antiserum in overlay on plaque number and plaque size. Cultures inoculated with 30 to 60 PFU of X-7 were incubated under overlays containing serial dilutions of two NWS or two RI/5$^+$ antisera or control sera. The control sera came from the preimmunization bleedings of the two rabbits immunized with RI/5$^+$ virus. The plaque number and median plaque radius in these plates are shown in Fig. 3.

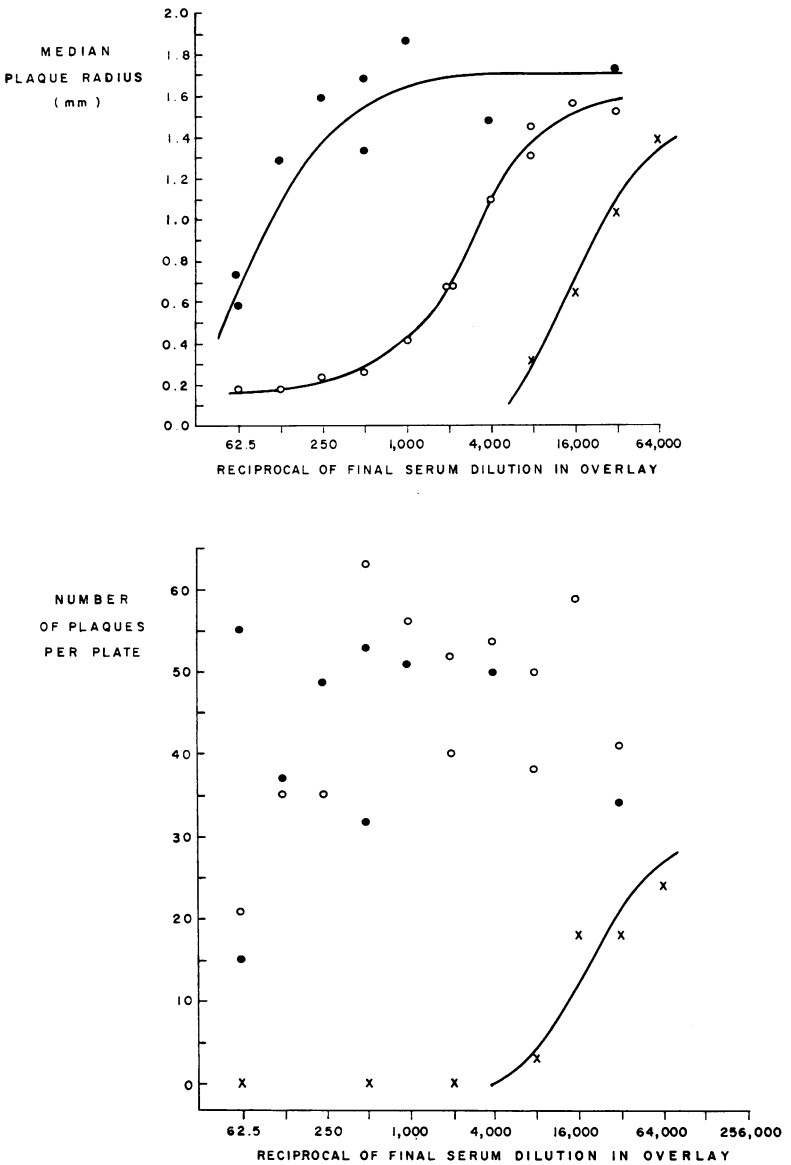

FIG. 3. *Effect of incorporation of serial serum dilutions in overlay on X-7 median plaque radius and number of plaques per plate. Symbols:* ●, *normal rabbit serum (R 231, preimmunization);* ○, *RI/5$^+$ antiserum (R 231, bled on 17th day after three injections);* ×, *NWS antiserum (R 223, bled on 45th day after three injections).*

Normal rabbit serum decreased plaque size up to dilutions of about 1:500 and reduced plaque number only at the lowest dilution used (1:62.5). The RI/5$^+$ antisera had no effect on plaque number at any dilution used, beyond that observed with normal serum. They did, however, reduce plaque size considerably more than did normal serum; the reduction was evident up to dilutions of 1:4,000. The NWS antisera produced an initial zone of complete plaque inhibition, followed by a narrow zone in which *both* size and number of plaques were reduced. Additional experiments showed that X-7 antiserum had the same pattern of effects on X-7 as did NWS antiserum and showed that NWS antiserum had the same pattern of effects on NWS virus as it did on X-7 virus. Experiments in which microscopic hemadsorption was used did not result in a change in the PI titer of NWS antiserum with NWS and X-7 viruses. Therefore, the zone of complete plaque inhibition in these systems is not a zone in which plaque size is reduced to a point where the plaques are too small to be detected by ordinary microscopic examination. Thus, occurrence of plaque size reduction without concomitant change in plaque number at any serum dilution (PSR pattern) in the X-7 virus-RI/5 antiserum system is a pattern of reactivity distinct from the change in plaque size and number (PI pattern), observed with X-7 virus and either X-7 (homotypic) or NWS antisera.

Kinetics of plaque growth of X-7 under overlays containing RI/5 antisera. PSR of X-7 after 4 days of incubation under RI/5 antiserum could have been due either to a continuously lower plaque growth rate during that period or to an initial inhibition of plaque development followed by a resumption of plaque growth in a manner analogous to the "breakthrough" phenomenon (20). To distinguish between these two possibilities, cultures inoculated with 50 to 100 PFU of X-7 were incubated under an overlay containing RI/5$^+$ antiserum or control serum in a final dilution of 1:500 and were fixed at daily intervals. The control serum was RI/5$^+$ antiserum adsorbed with RI/5$^+$ virus. The results are shown in Fig. 4 and 5. There was little difference between RI/5$^+$ antiserum and control serum in the variation in plaque number with time (Fig. 4). The low number of plaques in both plates on the 2nd day was associated with plaque sizes bordering the lower limit of discernibility. This experiment was performed without microscopic hemadsorption so that plaques with a radius smaller than 0.1 mm could not be differentiated from nonspecific holes in the monolayers. The median plaque radius with control serum and RI/5$^+$ antiserum varied with time according to a curve which was close to a straight line, particularly when corrected medians were used (Fig. 4b and c). Correction of the median was made on data from plates fixed on day 2, because the number of plaques

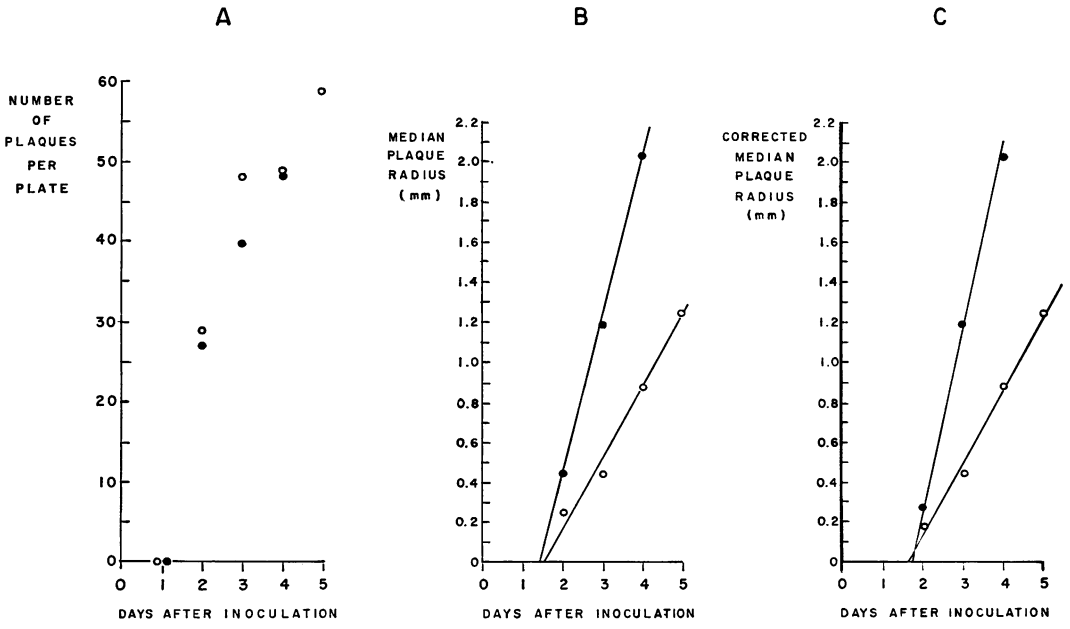

FIG. 4. *Variation in X-7 plaque number and median plaque radius with time after inoculation. Sera were incorporated in the overlay at final dilutions of 1:500. Symbols:* ○, *RI/5$^+$ antiserum (R 228, bled on 45th day after four injections);* ●, *control serum: above serum absorbed with RI/5$^+$ virus.*

was less than the number at the time of the plateau (approximated as 50). The corrected median plaque radius was calculated on the assumption that there were plaques too small to be seen, besides visible plaques, in such number as to make a total corrected number 50 plaques. The slope was less steep with RI/5$^+$ antiserum than with control serum. Extrapolation of the RI/5$^+$ antiserum and control serum lines suggests that both lines have a common intercept with the time axis at about 36 to 42 hr. [The median plaque radius represents the median radius of the areas occupied by necrotic cells which detached during washing and fixing. Hence, the intercept with the time axis represents the median time when the first cell in the plaque is sufficiently damaged to become detached on washing. This time interval fits well with the period of about 24 to 42 hr found for the same end point (detachment of one-half of the cells) when monolayers were infected with X-7 at a multiplicity close to 1.] Figure 5 shows the frequency distribution of X-7 plaque radius at different times after inoculation in plates overlaid with control and RI/5$^+$ antiserum; the mean plaque radius and the standard deviation are indicated in the figure. When the plaque size frequency distributions of plates under normal or RI/5 antiserum overlays fixed on the same day are compared, they appear to differ; but, when plates with similar means are compared, the frequency distributions and standard deviations are roughly similar. Thus, RI/5 antiserum decreases the growth rate of X-7 plaques without otherwise changing the plaque growth pattern. A second experiment, with B/Lee antiserum as control serum, gave similar results, except that the intercept was at 30 to 36 hr.

PSR with RI/5 antiserum as a stable, heritable property of X-7. X-7 virus was tested for PSR

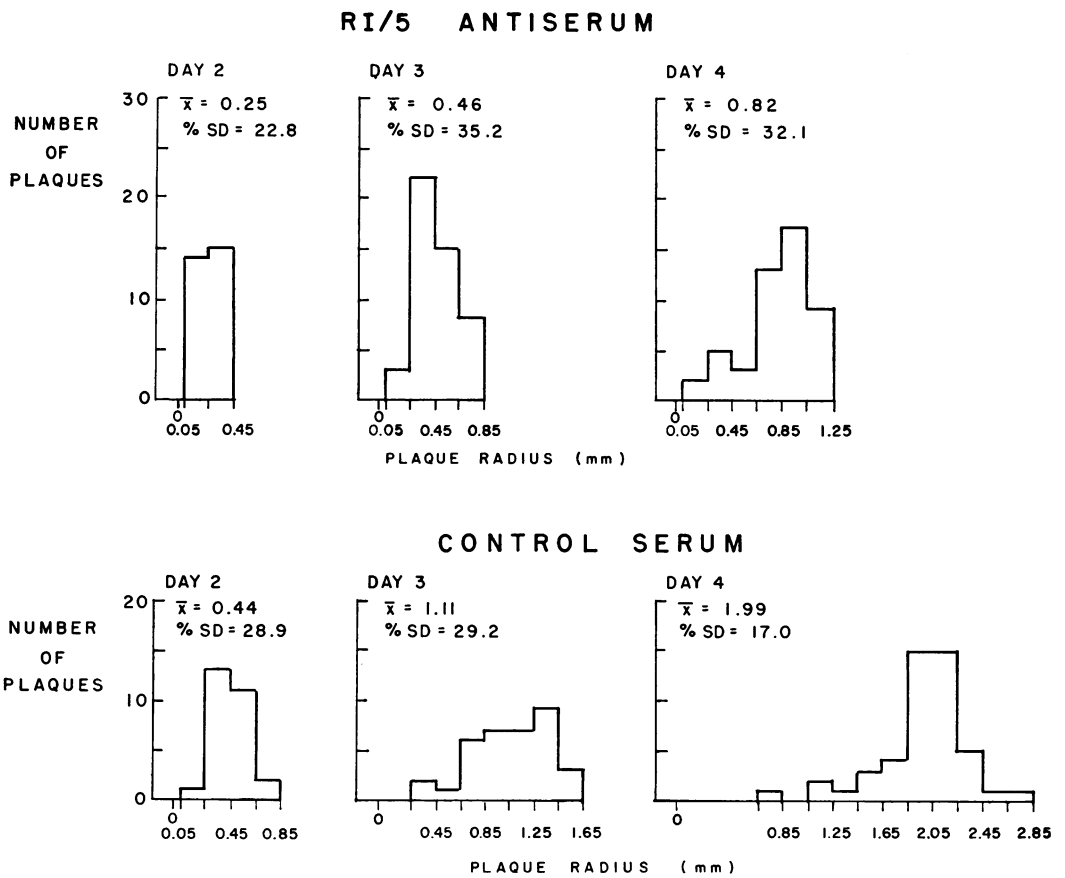

FIG. 5. *Frequency distribution of X-7 plaque radius with time after inoculation and with RI/5$^+$ serum or control serum in the overlay. Data derived from the same experiment as in Fig. 4. Sera and serum dilutions as in Fig. 4. Abbreviations: $\bar{x}$, mean plaque radius; % SD, standard deviation as percentage of mean plaque radius.*

with RI/5 antisera at various stages of allantoic and tissue culture passages and after recloning, as indicated in Fig. 1. The plaque size of X-7 with serumless overlays and the occurrence of PSR with RI/5$^+$ antisera were stable features during these transfers (Table 1). Virus recovered from X-7 plaques of reduced size in plates with RI/5$^+$ antiserum overlay did not differ qualitatively or quantitatively from X-7 either in its plaque size with serumless overlays or in its susceptibility to plaque size reduction with RI/5$^+$ antiserum overlay, as is illustrated by the following experiment (Fig. 6). Cultures inoculated with X-7 were incubated for 6 days under overlays either containing no serum or containing RI/5$^+$ antiserum. This prolonged incubation was chosen to obtain a better resolution of small differences in plaque size. One plaque near the median of the frequency distribution of plaque size (plaque a) was picked from a plate with serumless overlay, and two plaques (b and c), representative of the two extremes of plaque size, were picked from RI/5 antiserum plates. Virus from each of these three plaques was inoculated into two plates, one of which was incubated without serum and the other with RI/5$^+$ antiserum in the overlay. The frequency distribution of plaque size in these plates is illustrated in Fig. 6. All three plates with the serumless overlay showed the same plaque size frequency distribution, and all three plates with RI/5 antiserum overlay showed PSR of the same extent.

TABLE 1. *Recombinant 7 plaque size and plaque size reduction by RI/5 antiserum during allantoic and tissue culture passages*

Expt no.	Passage history of X−7[a]	Median plaque radius[b]		No. of plaques per plate[b]	
		NRS[c]	RI/5 AS[c]	NRS[c]	RI/5 AS[c]
1582	E2	1.47	0.56	82	138
1585	E3	2.03	0.87	48	49
1599	E3	1.31	0.27	32	63
1677	E4	1.52	0.75	43	32
1806	E4p3E1p1E3	0.94	0.32	162	155

[a] E = allantoic passage, p = plaque isolation in conjunctival cell culture (i.e., E4p3E1 . . . = 4 allantoic passages followed by 3 successive plaque isolations followed by one allantoic passage).

[b] Median plaque radius (millimeters) and plaque number measured in the same plate fixed after 4 days of incubation.

[c] RI/5 AS = RI/5 antiserum. R231, day 17, at a 1:500 dilution in experiments 1582, 1585, and 1599; R232, day 17, at a 1:1,000 dilution in experiment 1677; and R230, day 37, at a 1:1,000 dilution in experiment 1806. NRS = normal or virus-adsorbed rabbit serum.

PSR with X-7 as a regular and specific serological response of rabbits to RI/5 immunization. The plaque size reducing property of RI/5 antiserum can be removed by adsorption with RI/5 virus. This result suggests that X-7 plaque size reduction is caused by an antibody specific for an antigenic component(s) present in RI/5 virus. Additional studies with various antisera and adsorbing viruses support this concept (E. D. Kilbourne et al., *in press*). That this antigenic component is common to several RI/5 variants, including those sensitive and resistant to inhibitors, is shown by antiserum-in-overlay tests performed with X-7 and several RI/5 antisera (Table 2). These data show that the ability to cause PSR of X-7 is not an occasional serological response of individual rabbits, but a regular response of rabbits to RI/5 immunization. All RI/5 antisera exhibited a wide zone of plaque size reduction without change in plaque number, extending over a 16-fold or greater range of dilutions; the occurrence of this zone appeared to be independent of the PSR titer of the serum, since it was found with sera ranging in titer from 1:1,000 to 1:64,000. Thus, a marked dissociation of the PI and PSR end points with X-7 appears to be a regular feature of the serological response of rabbits to RI/5 immunization. Indeed, it is questionable whether any PI of X-7 has been demonstrated with RI/5 antisera; the only possible instance of a plaque inhibitory titer beyond that of normal serum was with the high titer LW5 serum (Table 2). Microscopic examination revealed plaque size reduction without decreased plaque numbers at a dilution of 4,000, and there were numerous microscopic plaques at dilutions of 2,000 and 500. However, the poor quality of the monolayers at these dilutions in this experiment precluded definitive interpretation.

The dissociation of PI and PSR end points could have been either a feature limited to the RI/5 antibody-X-7 virus system, or a property that RI/5 antibody could manifest, in the proper conditions, with any antigenically related virus. It was not possible to test these hypotheses with wild-type homologous viruses, since the RI/5 viruses available produced no plaques, or only extremely small plaques, in the conjunctival cell system; moreover, the occurrence of these plaques was sometimes erratic. However, unequivocal PI of a large plaque recombinant (X-1L) antigenically identical with the RI/5 viruses has been demonstrated with RI/5 antisera (E. D. Kilbourne et al., *in press*). Another recombinant, X-7 (F1), which was antigenically similar to X-7, except for exhibiting greater reactivity with RI/5 antisera in complement-fixation and mouse infection tests (E. D. Kilbourne et al., *in press*),

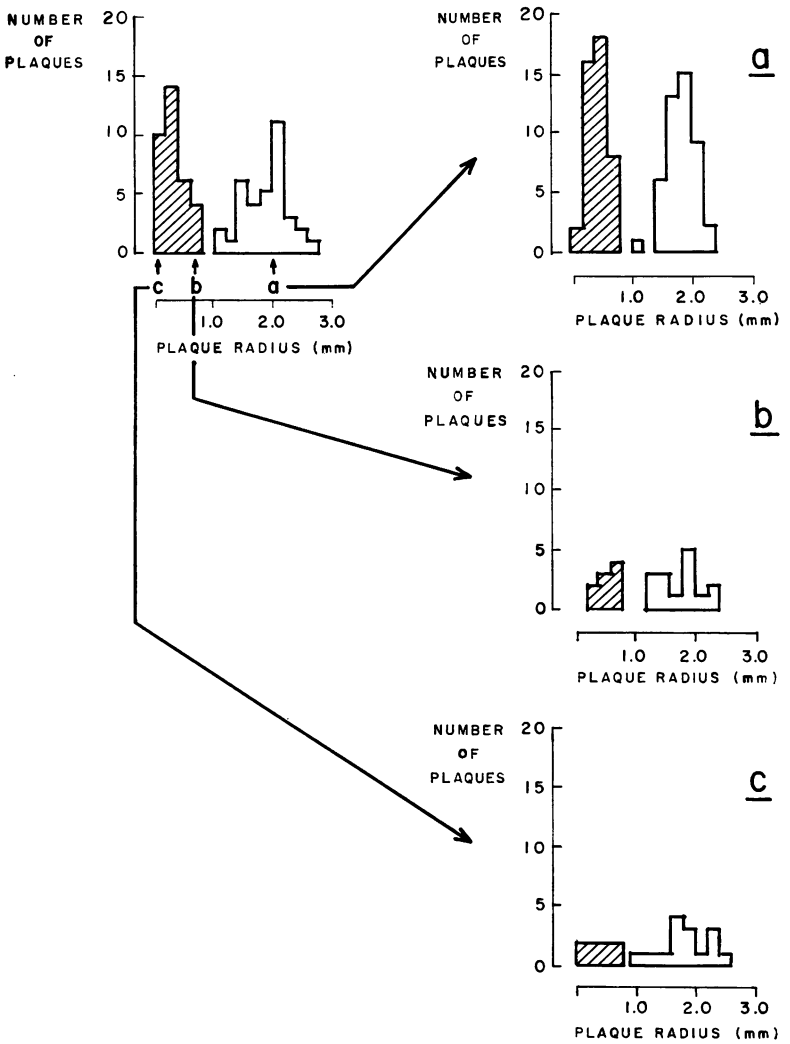

FIG. 6. *Plaque size reduction by RI/5+ antiserum of X-7 virus isolated from plaques of reduced size grown under RI/5+ antiserum overlay. Plaque size frequency distribution under serumless (empty bars) and RI/5+ antiserum (cross-hatched bars) overlays of X-7 and of its progeny virus picked from plaques developing under serumless and RI/5 antiserum overlays. The RI/5+ antiserum was that of R 232 bled on the 40th day after five injections. Histogram on left: parent virus (X-7, p1E5); the cultures were fixed on the 6th day and RI/5+ antiserum was used at a final concentration of 1:500. Histogram on right: (a) progeny virus obtained under serumless overlay, (b) and (c) RI/5+ antiserum overlay; the size of the plaques from which these viruses were picked is indicated by the letters and arrows on the histograms on the left; the cultures from which the histograms on the right were derived were fixed on the 5th day. Their overlay contained RI/5+ antiserum at a final dilution of 1:1,000.*

was also studied (Table 2). Unlike X-7, X-7 (F1) exhibited plaque size reduction with RI/5 antiserum only in close association with plaque number reduction. This result was obtained in two separate titrations. The PI and PSR titers of the antiserum were both 1:1,600 with X-7 (F1), and its PSR titer with X-7 was 1:2,000. Furthermore, adsorption with X-7 virus markedly decreased the PI titers of that serum against X-7 (F1). The assignment of a PI pattern to the X-7(F1)-RI/5 antiserum system must be qualified by the statement that microscopic hemadsorption has not been performed yet in this system. The possibility that the system exhibits extreme PSR pattern, giving the operational appearance of a PI pattern, is not yet completely excluded. These results indi-

TABLE 2. *PSR and PI patterns of X-7 and other viruses with different antisera*

Test virus	Immunization schedule				Immune serum		Control serum[a]	
	Immunizing antigen	Rabbit no.	No. of injections	Day bled	PSR	PI	PSR	PI
X-7	RI/5+	231	3	17	2,000[b]	62.5[b]	62.5	62.5
X-7	RI/5+	232	3	17	1,000	<62.5	<125	62.5
X-7	RI/5+	232	5	40	2,000	<62.5		
X-7	RI/5+	228	4	45	2,000	<400		
X-7	RI/5+	230	5	37	4,000	<62.5	62.5	<62.5
X-7	RI/5−	234	5	37	2,000	125		
X-7	RI/5−	LW5	2	47	64,000	<4,000	125	125
X-7	NWS-Mk-p	242	1	2	250	125	125	<125
X-7	NWS-Mk-p	242	1	4	16,000	16,000	250	<250
X-7	NWS 34	222	3	45	8,000	8,000		
X-7	NWS 34	223	3	45	16,000	16,000		
NWS	NWS 34	222	3	24	128,000	128,000		
NWS	NWS-34	222	3	45	16,000	16,000		
NWS	NWS 34	223	3	45	16,000	16,000		
X-1L	RI/5+	231	5	40	8,000	8,000		
X-1L	NWS	222	3	45	<1,000	<1,000		
X-7 (F1)	RI/5+	231	5	40	8,000	8,000		
X-7 (F1)	RI/5+	232	5	40	1,600	1,600	<400	<400
X-7 (F1)	NWS	222	3	45	>16,000	>16,000		

[a] This was the preimmunization serum of the same rabbit whose immune serum was used in that experiment, except in the case of rabbit LW5 when the preimmunization serum of another rabbit (LW9), also received from Australia, was used, and in the case of the experiment with X-7 (F1) when the control serum was the R232 immune serum after adsorption with X-7.

[b] Reciprocal of highest dilution showing 50% reduction in median plaque radius (PSR) and plaque number (PI).

cate that the dissociation of plaque size reduction from reduction in plaque number (PSR pattern) is a property exhibited by RI/5 antisera only with certain viruses like X-7, and that RI/5 antisera are capable of plaque size reduction in close association with plaque number reduction (PI pattern). They also show that X-7 can remove from RI/5 antisera an antibody capable of causing at least operational PI with another virus, X-7(F1). These data are consistent with the hypothesis that the same antibody is responsible for X-7 plaque size reduction and for X-7(F1) plaque inhibition. They do not exclude other interpretations.

PI with X-7 as a regular serological response of rabbits to NWS immunization. The contrasting pattern of close relationship of plaque size reduction with plaque number reduction was exhibited by X-7 with several different NWS antisera (Table 2). The use of microscopic hemadsorption usually revealed a few additional microscopic plaques, but considering those did not change the PI titers. NWS antisera PI titers with X-7 and with NWS were very close (Table 2).

Relation of time after immunization to the ability of a serum to produce PSR or PI. Other experiments were performed to find whether the ability of a serum to produce PSR or PI with X-7 virus was influenced by the time at which the serum is obtained after immunization. Rabbits were bled 4, 7, and 10 days after a single injection of RI/5+, RI/5−, and NWS viruses. The rabbits injected with NWS were also bled on the 2nd day. These sera, at a final dilution of 1:250, were incorporated in the overlay of plates inoculated with X-7. The only response observed with the RI/5 antisera was PSR; it was demonstrable in three of four 4th-day sera and in all four 7th- and 10th-day sera; the degree of plaque size reduction increased with time after injection. Plates overlaid with the second day NWS antisera showed decreased plaque size and plaque number when compared with preimmunization serum. Titration of one of the 2nd-day NWS antisera yielded PI and PSR end points of 125 and 250, respectively. These data represent either a more marked than usual nonspecific serum effect or the beginning of a specific PI pattern of response. Titration of one of the 4th-day NWS antisera showed a typical PI pattern with an end point of 16,000. Thus, the PI or PSR pattern of reactivity of a serum with X-7 is established very early in the course of immunization and does not change with time after immunization.

Reactivity of X-7 in other neutralization tests with RI/5 and NWS antisera. Other types of neutralization tests were performed to relate the PSR pattern of X-7 virus with RI/5 antiserum to other indices of viral antigen-antibody reaction. Three methods of "preinoculation" neutralization were used: serum-dilution preinoculation neutralization, preinoculation neutralization tests with concentrated virus, and a modified disc method.

Serum-dilution preinoculation neutralization. Samples of virus containing 30 to 60 PFU per 0.1 ml were mixed with equal volumes of serial dilutions of antiserum and allowed to react at room temperature for 30 min. Then 0.2 ml of the mixture was added to the cultures, adsorbed for 30 min, and washed with 10 ml of medium 199 before adding the overlay as usual. The results of serum-dilution preinoculation neutralization tests in conjunctival cell culture and in ovo are shown in Table 3. The NWS antisera neutralized X-7 and NWS viruses in cell culture and in ovo at high dilutions; the titers with X-7 viruses were very close to those obtained with NWS virus; the serum dilution titers obtained with these viruses in preinoculation neutralization tests were 2- to 32-fold lower than the titers of the same sera with the same viruses in the antiserum-in-overlay test (Tables 2 and 3). The RI/5$^+$ and RI/5$^-$ antisera neutralized RI/5$^-$ in cell cultures and in ovo at high dilutions, with titers comparable to those of the NWS antisera with their homologous viruses. However, RI/5 antisera showed neutralizing activity against X-7 only at very low dilutions — the titers varied between less than 4 and 16; i.e., they were 100-fold to more than 1,000-fold lower than their titers with the RI/5 homotypic virus, and more than 1,000-fold lower than the PSR titers of the same sera with X-7 (Tables 2 and 3). There were no prozones nor any evidence of enhancement of infectivity by antisera as described in other systems (3). These results point to another difference in the behavior of X-7 with antisera to its two parental viruses and also show a remarkable lack of parallelism between the results of PSR and of preinoculation neutralization tests in the X-7-RI/5 antiserum system.

Preinoculation neutralization tests with concentrated virus. In another type of preinoculation neutralization test (Fig. 7), samples of virus containing 10^7 PFU per 0.1 ml were mixed with equal volumes of 1:32 dilutions of serum and allowed to react at 4 C. At serial intervals, up to 3 hr, portions were removed, appropriately diluted for plaque assay, and inoculated as described above for the serum-dilution preinoculation neutralization test. With the NWS antiserum, the neutralization kinetics exhibited a biphasic curve, similar to the one described by Lafferty (8). The RI/5 antisera showed only a slow phase, if any, with a two- to three-fold reduction at most in PFU over a 3-hr period. Normal serum had no detectable effect under these conditions.

Modified disc method. To approximate more closely the environmental conditions under which PSR is demonstrated (temperature, agar and other components of the overlay, etc.), a modifi-

TABLE 3. *Preinoculation serum-dilution neutralization tests*

Antisera and immunization schedule				Neutralization of test virus					
Rabbit no.	Immunizing antigen	No. of injections	Day bled	X-7		RI/5[a]		NWS-MK-p	
				EN[b]	TCN[c]	EN	TCN	EN	TCN
231	None	—	—	2	2	<8	<32	—	—
232	None	—	—	—	<20	—	—	—	—
LW9	None	—	—	<4	<4	<4	—	<4	—
231	RI/5$^+$	3	17	16	4	500	4,000	—	—
232	RI/5$^+$	5	40	—	<20	—	—	—	—
LW5	RI/5$^-$	2	47	8	16	2,000	—	4	—
222	NWS 34	3	24	—	4,000	—	—	—	4,000
222	NWS 34	3	45	2,000	—	—	—	1,500	—
223	NWS 34	3	45	4,000	4,000	—	—	—	—

[a] The RI/5$^-$ strain was used in egg neutralization, and the RI/5$^-$ MK strain was used in tissue culture neutralization.

[b] EN=egg neutralization; reciprocal of serum dilution at which hemagglutination was detected in 50% of the eggs at a 1:4 dilution of allantoic fluid.

[c] TCN-tissue culture neutralization; reciprocal of serum dilution giving a 50% reduction in plaque number in conjunctival cell cultures.

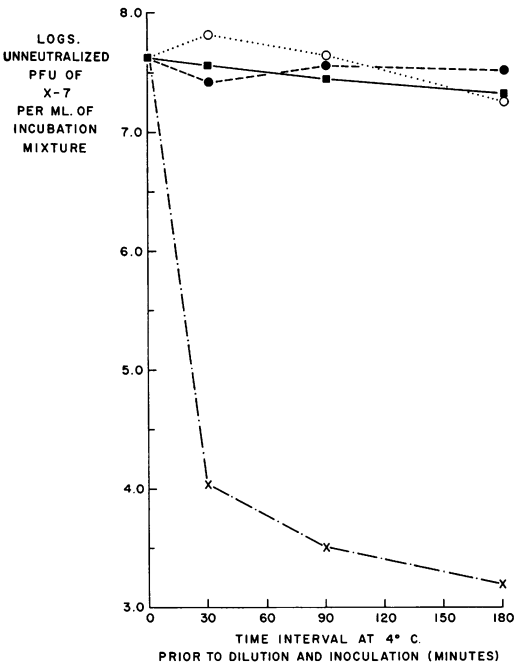

FIG. 7. *"Preinoculation" neutralization using 10^7 PFU of X-7 virus and 1:32 dilution of antiserum. Symbols: ■, medium 199; ●, normal rabbit serum (R231, preimmunization bleeding, PI and PSR titers with X-7 = 1:62.5); ○, RI/5+ antiserum (R231, bled on day 17 after three injections, PI titer with X-7 = 1:62.5, PSR titer with X-7 = 1:2,000); ×, NWS antiserum (R223, bled on day 45 after three injections, PI and PSR titer with X-7 1:16,000).*

cation of the disc method of Melnick and Benyesh-Melnick (11) was devised. Monolayers were covered with 2 ml of an overlay which contained normal rabbit serum, RI/5 antiserum, or NWS antiserum at a final dilution of 1:500. Six plastic cylinders (0.6 by 1 cm) were set on the overlay after it solidified, and 2 more ml of the same overlay was added outside the cylinders and allowed to solidify; one cylinder received 0.2 ml of medium 199, and the other five received serial dilutions of X-7 (Table 4). The plates were incubated for 5 days; the location of the monolayer underlying the cylinders was marked, and, after the overlay was removed and plates were stained, the number and size of the areas of cell necrosis were recorded. The virus added to the cylinders had to diffuse through the serum-containing overlay before reaching the cells; after the first cycle, the progeny were again, presumably, in contact with the serum in the overlay. This test thus has features of both "preinoculation" and "postinoculation" neutralization tests. A positive neutralization (i.e., no areas of necrosis) would not distinguish between neutralization that occurred before and after the first cycle, but a negative neutralization (i.e., no difference in occurrence of necrosis with control) would indicate at least failure to neutralize before the first cycle. The results are shown in Table 4. Three logs more virus were required in the presence of NWS antiserum than in the presence of normal serum for the induction of necrosis. There was less than 1 log unit difference between RI/5 antiserum and normal serum.

TABLE 4. *Reaction of RI/5 and NWS antisera with X-7 using the modified disc method*

Antiserum in overlay[a]	PFU of X-7 virus inoculated (log base 10)					
	5.6	4.6	3.6	2.6	1.6	None
NRS 231	C[b]	C	C, C	3, 4[c]	1, 0	0, 0
RI/5 231	C	C	C, 5	5, 1	0, 0	0, 0
NWS 223	C	1	0, 0	0, 0	0, 0	0, 0

[a] All sera incorporated in overlay at final dilution of 1:500. Sera from the same bleedings as those in Table 3. RI/5 231 had a PSR titer with X-7 virus of 1:2,000 and a PI titer of 1:62.5. NWS 223 had PSR and PI titers with X-7 virus of 1:16,000. NRS 231 (preimmunization bleeding of rabbit 231) had PSR and PI titers with X-7 virus of 1:62.5.

[b] Confluent areas of necrosis under the cylinder after 5 days of incubation.

[c] Number of discrete areas of necrosis under the cylinder after 5 days of incubation.

Thus, there is a discrepancy between the high reactivity of X-7 with RI/5 antiserum in the overlay with plaque size reduction as end point and its low reactivity, if any, with plaque inhibition as end point, in three types of preinoculation neutralization tests.

DISCUSSION

The reduction of plaque size of X-7 virus by RI/5 antiserum in the overlay has threefold interest. It is the end point of a simple test with which certain antigenic components of influenza recombinant viruses can be detected rapidly and easily; secondly, its study reveals a pattern of effects of virus-antibody reaction on plaque number and size which differs from that exhibited by other influenza virus-antibody systems; and lastly, it represents, as a result of a virus-antibody reaction, a modification of viral plaque pathogenesis that can be analyzed in terms of the antigenic constituents of influenza viruses and of their function in the virus-cell interaction.

The simplicity of the antiserum-in-overlay technique and its ability through PSR to pick up a clone of a new mutant or recombinant in the

same experiment in which it is detected have established it as a screening technique in this laboratory and have allowed the recognition and isolation of a variety of other recombinants (E. D. Kilbourne et al., *in press*). The results of quantitative studies of plaque size show that PSR of X-7 by RI/5 antiserum is not the result of selection of a virus with a smaller plaque size or decreased reactivity with RI/5 antiserum, since virus recovered from X-7 plaques of reduced size under RI/5 antiserum overlay has the same quantitative patterns of plaque size and plaque size reduction with RI/5 antiserum as does virus recovered from X-7 plaques under a serumless overlay. It has also been shown, by measurement of plaque size, that the reactivity of X-7 with RI/5 antiserum in the antiserum-in-overlay technique remains constant on allantoic passages of the virus. Thus, the same test can serve to recognize, isolate, and characterize a virus as a true and stable recombinant, initially and during subsequent passages.

The use of a recombinant virus in these experiments has shown that more than one pattern of plaque size reduction and plaque number reduction can occur as the result of virus-antibody reaction. The parental NWS and RI/5 rabbit antisera have two different patterns of reactivity with the NWS-RI/5 hybrid X-7 virus when incorporated into agar overlay in cell culture, namely, plaque size reduction closely associated with reduction in plaque number (PI pattern) and plaque size reduction without any demonstrable change in plaque numbers over a wide range of serum dilutions (PSR pattern). A small increment in the concentration of NWS antibody causes a shift from no effect on X-7 plaques to complete inhibition; the failure to detect any plaques at these dilutions, even with microscopic hemadsorption, indicates that NWS antiserum prevents the development of X-7 plaques by preventing the occurrence of the second cycle or of the early part of the multicyclic process leading to plaque formation. The simplest hypothesis to account for this is that virus liberated by the initially infected cell is irreversibly neutralized and that this process continues for the duration of the period of observation, or until the infected cell stops releasing virus. Interpretation of the effect of RI/5 antiserum must consider the kinetics of X-7 plaque growth in the presence of RI/5 antiserum; i.e., the variation in plaque radius with time remains linear but with a lower slope than with the control serum. These results indicate that RI/5 antibody acts on X-7 throughout the period of observation, and its effect at any given time is to reduce plaque growth to a constant fraction of the control plaque growth. The results of the antiserum-in-overlay titration indicate that X-7 plaque size growth rate is sensitive to the amount of RI/5 antibody.

Hypothetical mechanisms for plaque size reduction of X-7 by RI/5 antiserum must take into account the results of other immunological reactions besides those demonstrated with the antiserum-in-overlay systems. Several tests have shown no or, at most, equivocal "preinoculation" neutralization of X-7 virus by RI/5 antiserum. Electrophoretic analysis of the proteins of X-7 has shown that the only demonstrable component shared by X-7 and RI/5 viruses is neuraminidase (W. G. Laver and E. D. Kilbourne, Virology, *in press*). Present evidence suggests the antigenic identity of X-7 and RI/5 neuraminidases (Laver and Kilbourne, *in press*).

These data are consistent with the following hypothesis. RI/5 antibody cannot neutralize X-7 virus before the first cycle of viral multiplication. Its reaction with X-7 neuraminidase on or at the surface of the infected cells decreases the yield, at each cycle, by a constant fraction or increases the mean latent period. This hypothesis fits all experimental facts, as well as the concept of virus-specified antigenic changes at the surface of influenza infected cells (2) and recent evidence suggesting that neuraminidase plays a role in influenza virus release (13).

Another hypothesis, based on the idea that the significant feature of the RI/5 component of X-7 viral protein is that it is a "minor" antigenic component, has been presented elsewhere (6). This hypothesis interprets plaque size reduction in terms of Lafferty's model of influenza virus neutralization (9). It postulates that X-7 has a majority of closely spaced NWS determinants, allowing bridging by NWS antibody between sites on the same virus particle, and has few accessible RI/5 determinants, allowing bridging by RI/5 antibody only between sites on two different virus particles. The latter reaction would cause agglutination of X-7 particles and contribute to plaque size reduction by decreasing the number of infectious units and by slowing diffusion. The results presented here do not rule out this hypothesis but point to the need for additional assumptions to reconcile it with the discrepancy between the preinoculation and antiserum-in-overlay tests.

The results obtained with the RI/5-X-7 backcross, X-7(F1), show that, irrespective of whether or not the reaction of X-7(F1) with RI/5 antiserum is interpreted as PI or as an extreme PSR pattern, it is clear that the X-7(F1) (backcross) virus has more A_2 reactivity than X-7. This variation in reactivity of minor component is reminiscent of the results found with comple-

ment-fixation with A_2-avian influenza virus hybrids (17). This suggests that the antiserum-in-overlay technique may be a useful tool to study quantal changes in the antigenicity of influenza viruses. Systems involving other recombinants should be studied to find whether it is possible to demonstrate gradations in PSR (or in PI) when the same antiserum acts on different viruses. It is essential for such studies that full advantage be taken of the quantitative features of plaque size reduction, through use of microscopic hemadsorption, precise measurement of plaque size, and a range of serum dilutions.

It is not known how general is the occurrence among other viruses of the two patterns of reactivity under antiserum-containing overlays described here, nor whether they represent two separate classes or two extrema separated by numerous intermediaries. The pattern exhibited by the X-7 virus-RI/5 antiserum system (dissociation of plaque size reduction and plaque number reduction over a wide zone of serum dilutions) is not unique to this system; it has been found with other influenza recombinant viruses (E. D. Kilbourne et al., *in press*). The PSR pattern has not yet been found in systems which do not involve recombinant viruses; however, very few nonrecombinant influenza viruses and no viruses other than influenza have been studied in this laboratory with the antiserum-in-overlay technique. Therefore, one cannot state at present whether the PSR pattern is unique to recombinant viruses, to influenza viruses, to all viruses possessing a neuraminidase, or to viruses which exhibit virus-directed antigenic changes at the surface of infected cells, or whether it is a general phenomenon among viruses. Wecker (19) and others have demonstrated the occurrence of plaque size reduction without reduction in plaque numbers in certain poliovirus systems, but extensive studies of the effect of serum dilutions and microscopic study of "negative" plates have not been reported. Thus, it cannot be concluded whether the instances of plaque size reduction without reduction in plaque number in the poliovirus systems are equivalent to our PSR pattern, to an intermediary pattern, or to the end point dilution in a PI pattern.

An ancillary aspect of this work is the demonstration of another system in which plaque growth kinetics appear to be linear with respect to plaque radius. There are now several such systems, including bacteriophage (7), vaccinia (10), foot-and-mouth disease (15) and certain echo 6 strains (1). The X-7 virus-RI/5 antiserum system provides a new method for decreasing the slope of the plaque radius growth curve at will through change of the antiserum concentration.

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