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Fate of Rubella Genome Ribonucleic Acid After Immune and Nonimmune Virolysis in the Presence of Ribonuclease

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To determine whether rubella virion ribonucleic acid (RNA) becomes accessible to nuclease attack after immune lysis of the viral envelope, virions containing radioactively labeled RNA were examined in three ways with the following results. (i) Incubation of purified virus with heat-inactivated rubella convalescent human serum and guinea pig complement resulted in an increase in acid-soluble RNA. Antibody was required; the reaction was temperature dependent and was blocked by ethylenediaminetetraacetic acid. When exogenous nuclease was added prior to lysis, radioactivity in virions was reduced to 15% of that in unlysed control pellets. (ii) Sucrose gradient sedimentation profiles of RNA released from lysed and unlysed virions under controlled conditions showed that the nuclease content of serum-virus mixtures was sufficient to eliminate all RNA of genome size, although degradation was not complete. (iii) Virions were also lysed by unheated human immune sera in the absence of guinea pig complement and by some, but not all, unheated antibody-negative sera.

When rubella virus is incubated with unheated convalescent serum, holes that can be seen by electron microscopy are produced in the virion envelope (1). The lesions resemble those produced on erythrocyte surfaces by the action of complement (4) and, it was reasoned, might permit access of ribonuclease (RNase) to the enzyme-sensitive nucleocapsid core (9). If this were true, not only would it provide one possible explanation for the enhancement of rubella virus neutralization by complement (15), but it would also provide an in vitro method for studying rubella virolysis and the antigens and antibodies that combine to initiate the process. To test this hypothesis, rubella virions containing radioactively labeled ribonucleic acid (RNA) were incubated with human convalescent serum, complement, and RNase; the integrity of the RNA was then examined. Under these conditions RNA became acid soluble and few virions remained intact. This procedure was then used to compare the lytic activity of immune and nonimmune sera.

MATERIALS AND METHODS

Virus. The Therien strain of rubella virus isolated in this laboratory in 1964 was grown in Vero cells as previously described (12).

Radioactive labeling of virion RNA. On the first day that hemagglutinin was detected in infected cultures, medium was replaced. After 4 h, actinomycin D (5 μg/ml, Merck, Sharpe and Dohme) was added, and 30 min later, [5-^{3}H]uridine (50 μCi/ml, New England Nuclear Corp.) was added. After a 24-h labeling period the medium was harvested, clarified by low-speed centrifugation, and layered over a 40-ml gradient cushion of sucrose (30 to 65%, wt/wt). The medium was centrifuged in an SW25.2 rotor for 18 h at 22,500 rpm, and fractions were collected through the bottom of each tube. The virion fractions were located by hemagglutinin content, pooled, and rebanded by sucrose gradient centrifugation in an SW41 rotor for 18 h at 35,000 rpm. The visible virion band was removed through the side of the tube with a syringe and needle and stored at −70°C.

Sera. Human sera collected before and after natural and vaccine-induced infection were used. Rubella hemagglutination-inhibiting (HI) antibody titers for each sample had been determined as described previously (11). The serum chosen for use as a positive standard (M.G.) was obtained 45 days postonset and had an HI titer of 128 (immunoglobulin M: 32) and a complement-fixing titer of 32. Antibody-negative sera consisted of pre-illness or prevaccination specimens and a pool of 10 rubella HI-negative sera. All were heated at 56°C for 30 min before use unless specified otherwise.

Standard test for virolysis. Equal volumes (20 to 200 μl) of virus, antiserum, and guinea pig complement (BBL, Division of Becton, Dickinson & Co.) were incubated overnight at 4°C. RNase A (Worthington Biochemicals Corp.) was then added to designated tubes at a final concentration of 60 μg/ml, which provided an excess of nuclease activity to obviate variations in endogenous serum levels (5, 6). At the end of a 2-h incubation period at 37°C, the contents of each tube were transferred by successive washings to tubes for the SW50.1 rotor containing a

2-ml cushion of 20% (wt/wt) sucrose. Virions were pelleted at 40,000 rpm for 2 h at 4°C, and the acid-insoluble radioactivity in each pellet was measured.

Measurement of radioactivity. Virion pellets were resuspended in 100 μl of 5% trichloroacetic acid with 140 μg of carrier transfer RNA (Miles Laboratories, Inc.) and held at 4°C for 1 h. Precipitates that developed were collected by centrifugation at 13,000 × g for 5 min; the pellets were then suspended in 400 μl of Nuclear Chicago solubilizer (NCS) (Amersham/Searle) and incubated at room temperature for 30 min. The resulting samples were then added to 5 ml of Spectrofluor (Amersham/Searle) and counted in a Beckman model LS-150 scintillation counter to an accuracy level of at least 5% after nonradioactive excitation of the fluors had fully subsided (1 to 2 days). To determine acid-soluble radioactivity, portions of the supernatants from acid precipitation were mixed with 4 volumes of NCS and incubated at room temperature for 30 min before mixing with the scintillation fluid.

Estimation of lytic efficiency. Sera were compared with the standard serum (M.G.), which was tested after inactivation at 56°C both with heated (negative control) and with unheated (positive control) guinea pig complement. The difference between the counts per minute in the positive and negative control pellets was considered to represent a 100% efficient reduction in acid-insoluble radioactivity, and this amounted to 800 to 1,500 cpm in the different experiments. The decrease in pelleted RNA after incubation with a given serum was reported as a percentage of the positive control.

RESULTS

Conditions for lysis. Purified rubella virus was incubated with the standard heat-inactivated human convalescent serum and RNase A for different lengths of time under conditions that permit or block the action of complement. It can be seen from Fig. 1 that lysis leading to exposure of genome RNA to the degradative action of RNase occurred only in the tube that contained unheated guinea pig serum and no ethylenediaminetetraacetic acid (EDTA). RNA degradation reached a maximum between 1 and 2 h of incubation at 37°C; the reaction did not proceed at 4°C.

Distribution of acid-soluble and -insoluble radioactivity in reaction mixtures and in virion pellets. To obtain a more complete picture of the specificity of the reaction and the extent to which virion RNA would be degraded in the absence of exogenous RNase, both acid-soluble and -insoluble radioactivities were measured in reaction mixtures and in the virion pellet fractions, as shown in Table 1. Seventy percent of

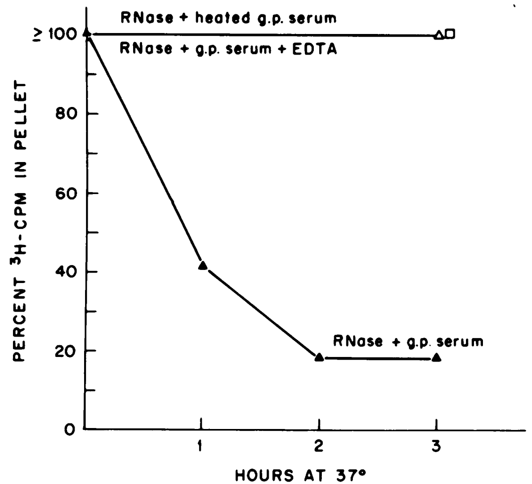

FIG. 1. *Radioactivity ([5-³H]uridine) in virion pellets after incubation with undiluted human rubella convalescent serum, undiluted guinea pig serum, and RNase A at a final concentration of 60 μg/ml under conditions that favor or block virolysis. EDTA was used at a concentration of 0.02 M to block the reaction.*

TABLE 1. *Acid-soluble and -insoluble radioactivity (^{3}H cpm) in rubella virus-antibody mixtures and in the virion-pellet fraction after incubation with guinea pig serum (C′) in the presence and absence of RNase*[a]

Sample	Radioactivity (^{3}H cpm)					
	4°C C′	37°C C′	Difference from control	37°C heated C′ + RNase	37°C C′ + RNase	Difference from control
Whole mixture	A	B	B − A	C	D	D − C
Acid insoluble	3,769[a]	3,344		3,360	1,764	
Acid soluble	1,639	2,291		2,316	3,601	
Increase in acid-soluble radioactivity			652			1,285
Virion pellet	a	b	a − b	c	d	c − d
Acid insoluble	2,061	1,953		2,320	317	
Acid soluble	220	468		95	25	
Decrease in total radioactivity in pellet			0			2,073

[a] Standard convalescent serum M.G. was used undiluted.

the radioactivity in control mixtures held at 4°C was acid insoluble, and 55% of this was recovered in the virion pellet. Most of the acid-soluble radioactivity in the reaction mixtures can be accounted for by diffusion of unincorporated [^{3}H]uridine down into the virion band region during the centrifugations, thus giving a moderately high background level. This, of course, was not found in the virion pellet fraction.

Some RNA was rendered acid soluble after incubation with RNase at 37°C in the absence of complement (Table 1, A − C). However, acid-soluble radioactivity in the virion pellet was comparable to that of the 4°C control (Table 1, a − c). This indicates that the unlysed virions that were sedimented under the conditions of the experiment were intact and that the genome was protected from the action of RNase. This can be visualized in the electron micrograph of negatively stained virions pelleted from a buffer control (Fig. 2a). The particles appear full and have regular borders; particles incubated with antibody and complement, on the other hand, were few in number and showed lesions in their envelopes and a halo of immune reactants (Fig. 2b).

Recovery of radioactivity in virion pellets from 37°C mixtures that contained active complement but no added RNase was comparable to that of 4°C controls (Table 1, a − b), with a very slight increase in acid-soluble counts. In the presence of exogenous RNase, however, only 14% (range 6 to 20%) of the acid-insoluble radioactivity in the matched control was recovered in the pellet (d/c × 100). Sixty-two percent of the RNA which after the reaction failed to pellet was converted to acid-soluble RNA detectable in the unfractionated mixture (D − C).

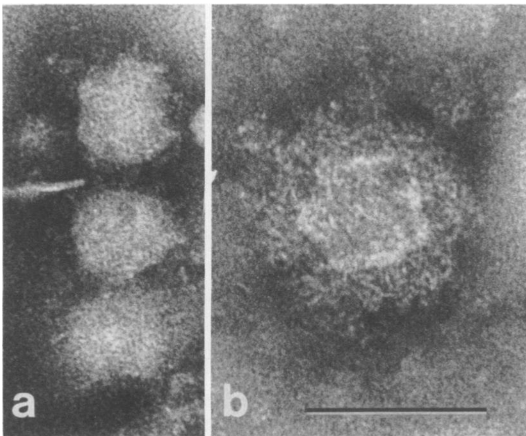

FIG. 2. *Phosphotungstic acid-stained rubella virus particles. Bars represent 100 nm. (a) Untreated particles; (b) particle incubated with antibody and complement.*

Immunological specificity of the reaction. Table 2 shows that when heat-inactivated serum was used, lysis did not proceed in the absence of rubella antibody. The standard convalescent serum, which had a rubella HI titer of 128, was effective at a dilution of 1:125 but only partially effective at a dilution of 1:625. Although in the natural disease lytic efficiency tended to parallel HI titer, this was not the case in vaccinees receiving HPV_{77} DE. This disparity is illustrated best by the 2-month postvaccination serum 1328-2A.

Sedimentation profiles of RNA from unlysed virions and from virions lysed in the presence and absence of exogenous RNase. The data in Table 1 show that some RNA in the reaction mixtures was rendered acid soluble without the addition of exogenous RNase A, although, as noted above, at least 95% of the radioactivity that pelleted with the virus remained acid insoluble. To determine the integrity of this RNA as compared with RNA from untreated virions, sodium dodecyl sulfate and EDTA were added to reaction mixtures at the end of the incubation period, and the released

TABLE 2. *Acid-insoluble radioactivity ([^{3}H]uridine) in virions pelleted after interaction with guinea pig complement, RNase, and heat-inactivated human serum that either contains or lacks rubella antibody*

Serum	Description	Dilution	HI titer	% RNA in virion pellet
M.G.	Convalescent 45 days postonset	1:1	128	100[a]
M.G.		1:1	128	16
		1:5		19
		1:25		6
		1:125		23
		1:625		76
14816	Pre-illness	1:1	<8	88
	3 wk postonset	1:1	16	30
109-2A	Prevaccination[b]	1:1	<8	92
	2 mo postvaccination	1:1	32	45
	3 yr postvaccination	1:1	8	39
1328-2A	Prevaccination[b]	1:1	<8	102
	2 mo postvaccination	1:1	256	61
	3 yr postvaccination	1:1	64	66
Pool J	Pool of 10 rubella-negative sera	1:1	<8	95

[a] Guinea pig serum heat inactivated to provide negative control and 100% value.

[b] HPV_{77} DE (Merck, Sharp and Dohme).

RNA was then examined by velocity centrifugation. In addition, micrococcal nuclease was used as the exogenous enzyme in another tube, its action being blocked by EDTA before dissolution of the virions. It can be seen from Fig. 3 that there was sufficient nuclease activity in virus-serum mixtures to completely eliminate RNA of the 40S genome size, although as indicated by the shaded area and as shown in a previous experiment, most of the RNA was not made acid soluble under these conditions. When micrococcal nuclease was present during lysis, the RNA was completely degraded and all of the counts were found at the top of the gradient.

Fate of rubella genome RNA in virions incubated in unheated immune and nonimmune sera in the presence of RNase. Unheated human sera that had been stored for various lengths of time at −20°C were tested for their ability to induce lysis. Each serum was compared with the standard serum (M.G.) as described above. As shown in Table 3A, immune sera that had been stored for 2 weeks or less induced virolysis, with efficiencies ranging from 30 to 81%. Incubation of virions in the standard convalescent serum without the addition of exogenous complement or RNase resulted in elimination of pelletable RNA at an efficiency of 80%. Three of the six antibody-negative sera also induced appreciable lysis in the absence of guinea pig complement. The length of time that a serum had been stored did not appear to be critical, as one serum stored for only 3 days at −70°C was less lytic than a serum from the same individual collected 8 months previously and stored at −20°C.

To obtain more data regarding the potential of unheated normal human sera to sensitize rubella virions for complement lysis, prevaccination sera from serologically confirmed rubella susceptibles were tested. To ensure that sufficient complement was present in this case, fresh guinea pig serum was added (Table 3B). Five of the nine sera tested were almost as effective (≥75%) as the rubella convalescent standard, and all appeared to induce some lysis.

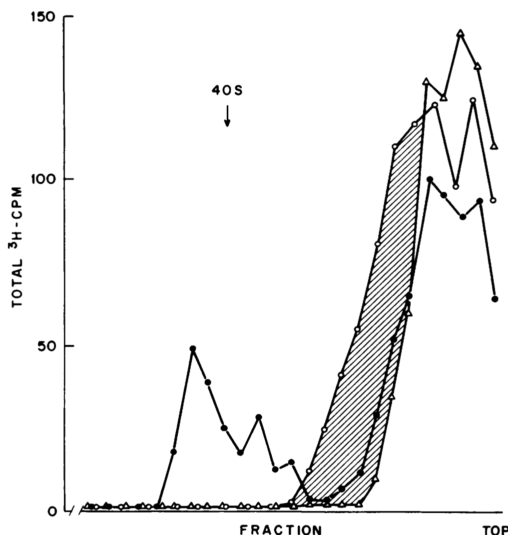

FIG. 3. *Sedimentation profiles of RNA released from rubella virions after incubation at 37°C with buffer (●) or with convalescent immune serum and complement without micrococcal nuclease (○) and with nuclease added (△). Reaction mixtures were adjusted to contain 2% sodium dodecyl sulfate and 0.02 M EDTA, and 0.2 ml was then layered over a 15 to 30% (wt/wt) sucrose gradient containing 0.2% sodium dodecyl sulfate, 0.001 M EDTA, and 0.1 M NaCl. The RNA was centrifuged at 51,000 rpm for 90 min at 4°C in an SW50.1 rotor. The shaded area indicates radioactivity that would be acid precipitable.*

DISCUSSION

Since Berry and Almeida first showed morphological evidence for immune virolysis (3), there have been only a few studies dealing with this process (1, 2, 13, 14, 17, 18). This seems surprising in view of the potential it offers for the study of virion membrane structure and immune reactivity.

Certain technical considerations were found to be important during our study. Manipulations of virus such as freezing and thawing must be kept to a minimum to reduce nonspecific damage to the envelope which would permit access of nuclease. It is also essential to employ a tissue solubilizer to overcome the quenching effects of serum which, if unrecognized, can give results which are difficult to interpret (17). The problem of quenching is particularly severe when virolysis is measured by following the increase in acid-soluble RNA in unfractionated serum-virus mixtures. To ensure unambiguous results, we chose to pellet virions before measuring radioactivity.

The experiments described indicate that serum nucleases, acting in concert with complement to eliminate infectious nucleic acid, may play an important role in defense (both immune and nonimmune) against viruses like rubella. In a preliminary report of their studies of RNase and DNase levels in human sera, Herriott et al. postulated in 1961 that such might be the case (8) and, in subsequent studies, nuclease concentrations in healthy adults were

TABLE 3. *Virolytic activity of unheated human sera stored at −20°C*

Serum	History	Rubella HI titer	Period of storage	Guinea pig C′	RNase A	Efficiency[a] of lysis (%)
(A)						
M.G. (heated)	Convalescent	128	>5 yr	+	+	100%
M.G.	Convalescent	128	>5 yr	−	−	80
15224	Unknown	64	2 wk	−	+	81
15227	Unknown	64	2 wk	−	+	30
15229	Unknown	128	2 wk	−	+	79
15231	Unknown	64	2 wk	−	+	56
14816	Pre-illness	<4	10 mo	−	+	21
LK 050875	Unknown	<8	8 mo	−	+	76
LK 011676	Unknown	<8	31 d (−70°C)	−	+	33
15013	Unknown	<4	2 mo	−	+	13
15033	Unknown	<4	2 mo	−	+	85
15095	Unknown	<4	1 mo	−	+	5
(B)						
1566	Prevaccination[b] susceptible	<8	>5 yr	+	+	87
1839	Prevaccination[b] susceptible	<8	>5 yr	+	+	85
1887	Prevaccination[b] susceptible	<8	>5 yr	+	+	79
1892	Prevaccination[b] susceptible	<8	>5 yr	+	+	57
62-1A	Prevaccination[b] susceptible	<8	>5 yr	+	+	90
723-1A	Prevaccination[b] susceptible	<8	>5 yr	+	+	55
1003-1A	Prevaccination[b] susceptible	<8	>5 yr	+	+	33
1031-1A	Prevaccination[b] susceptible	<8	>5 yr	+	+	27
1123-1A	Prevaccination[b] susceptible	<8	>5 yr	+	+	77

[a] Standard heated convalescent serum (M.G.) plus guinea pig complement considered to be 100% efficient.
[b] All seroconverted.

determined (5, 6). We have shown that sufficient endogenous RNase activity was present in serum-virus mixtures to degrade at least partially all rubella RNA of genome size exposed by virolysis (Fig. 3). An unexpected finding was that immunologically damaged virions with partially degraded RNA remained sufficiently intact so that essentially all radioactivity was recovered in virion pellets (Table 1, pellet b). Only when a high concentration of exogenous RNase was present that completely degraded the genome did disintegration of the particles ensue.

Heat-inactivated sera must be used to study antibody sensitization of virions, since some unheated antibody-negative sera will also induce lysis. However, when heated sera are used, the system provides a specific and sensitive tool for the study of membrane antigens. In studies to be published, we have found immune virolysis to be well correlated with complement-dependent neutralization. An antibody other than the HI antibody appears to be critical for lysis, but the latter is also required. Postvaccination sera with good HI titers may be relatively inefficient in sensitizing rubella virus as shown in Table 2, which suggests a deficiency in a necessary antibody.

The variability of the lytic capacity of unheated sera from rubella susceptibles suggests that natural defenses, not involving specific antibody, may also vary, not only between individuals but also at different times in the same individual. The stimulating factors and pathways of complement activation will be of interest in this context. In view of the demonstrated ability of C-reactive protein to activate complement (10, 16), it is perhaps noteworthy that an elevation in that protein was found in serum LK 050875, but not in LK 011676 drawn at a later date from the same person (Table 3A). Nonimmune lysis of C-type viruses has recently been described (18), and in a follow-up report evidence is presented that C1q in human serum interacts directly with the virion envelope, with subsequent activation of complement along the classical pathway (7).

The mechanisms of both immune and nonimmune virolysis are currently being investigated in our laboratory. We have demonstrated a method that can be applied effectively to elaborate on these biologically important processes in relation to rubella virus.

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LITERATURE CITED

1. **Almeida, J. D., and G. D. Laurence.** 1969. Heated and unheated antiserum on rubella virus. Morphological effect. Am. J. Dis. Child. 118:101-106.
2. **Almeida, J. D., and A. P. Waterson.** 1969. The morphology of virus-antibody interaction. Adv. Virus Res. 15:307-338.
3. **Berry, D. M., and J. D. Almeida.** 1968. The morphological and biological effects of various antisera on avian infectious bronchitis virus. J. Gen. Virol. 3:97-102.
4. **Borsos, T., R. B. Dourmashkin, and J. H. Humphrey.** 1964. Lesions in erythrocyte membranes caused by immune haemolysis. Nature (London) 202:251-252.
5. **Connolly, J. H., R. M. Herriott, and S. Gupta.** 1962. Deoxyribonuclease in human blood and platelets. Br. J. Exp. Pathol. 43:392-401.
6. **Connolly, J. H., R. M. Herriott, and S. Gupta.** 1962. Ribonuclease in normal and uraemic human blood. Br. J. Exp. Pathol. 43:402-408.
7. **Cooper, N. R., R. M. Welsh, F. Jensen, and M. B. A. Oldstone.** 1975. Proceedings VI International Complement Workshop, Sarasota, Florida.
8. **Herriott, R. M., J. H. Connolly, and S. Gupta.** 1961. Blood nucleases and infectious viral nucleic acids. Nature (London) 189:817-820.
9. **Hovi, T.** 1972. Release of rubella virus ribonucleic acid from nucleoprotein by polyanions. J. Virol. 9:879-882.
10. **Kaplan, M. H., and J. E. Volanakis.** 1974. Interaction of C-reactive protein with the complement system. I. Consumption of human complement associated with the reaction of C-reactive protein with pneumococcal C-polysaccharide and with the choline phosphatides, lecithin and sphingomyelin. J. Immunol. 112:2135-2147.
11. **Liebhaber, H.** 1970. Measurement of rubella antibody by hemagglutination inhibition. J. Immunol. 104:826-834.
12. **Liebhaber, H., J. T. Riordan, and D. M. Horstmann.** 1969. Growth of high titered rubella virus in roller bottle cultures of Vero cells. Proc. Soc. Biol. Exp. Med. 130:12-14.
13. **Oroszlan, S., and R. V. Gilden.** 1970. Immune virolysis: effect of antibody and complement on C-type RNA virus. Science 168:1478-1480.
14. **Radwan, A. I., D. Burger, and W. C. Davis.** 1973. The fate of sensitized equine arteritis virus following neutralization by complement or anti-IgG serum. Virology 53:372-378.
15. **Rawls, W. E., L. Desmyter, and J. L. Melnick.** 1967. Rubella virus neutralization by plaque reduction. Proc. Soc. Exp. Biol. Med. 124:167-172.
16. **Siegel, J., R. Rent, and H. Gewurz.** 1974. Interaction of C-reactive protein with the complement system. I. Protamine-induced consumption of complement in acute phase sera. J. Exp. Med. 140:631-647.
17. **Stollar, V.** 1975. Immune lysis of sindbis virus. Virology 66:620-624.
18. **Welsh, R. M., N. R. Cooper, F. C. Jensen, and M. B. A. Oldstone.** 1975. Human serum lyses RNA tumour viruses. Nature (London) 257:612-614.