ANALYSIS OF THE DEFECTIVENESS OF ROUS SARCOMA VIRUS, II. SPECIFICATION OF RSV ANTIGENICITY BY HELPER VIRUS[*, †]*

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The high-titer strain of Rous sarcoma virus (RSV) is defective, in the sense that it is unable to spawn infectious progeny without the intervention of a helper virus.[1] The first helper virus to be discovered was isolated from a stock of the high-titer strain of RSV, and has been named Rous associated virus, or RAV.[2] RAV has all the physical and biological attributes of viruses of the avian leukosis complex and is therefore considered a member of this group of viruses. In keeping with this classification, all avian leukosis viruses tested to date have been found to be active as helper viruses for RSV.

The precise role of helper virus in activating RSV has not yet been defined, but some possibilities for the helper function can be eliminated by considering those functions which RSV can carry out alone. Thus, RSV can effect the malignant transformation of chick embryo cells to sarcoma cells without the aid of helper virus. The sarcoma cells retain their characteristic morphology through many divisions. Since the transformed cells produce no mature RSV, they are called nonproducer, or NP, cells. If at any time during extended periods of cultivation, NP cells are superinfected with helper virus, a high proportion, if not all, of the NP cells will produce RSV. This demonstrates that the RSV *genome* is replicated in the NP cells without the aid of helper virus and that the helper virus is required only for the *maturation* of RSV. The most characteristic feature of virus maturation is the enclosure of the viral genome by an outer coat of protein or lipoprotein, which endows the virus with the property of infectiousness. The failure of the replicating RSV genome to mature into infectious virus suggests that the defect of RSV derives from its inability to direct synthesis of the virus-specific portion of its own outer coat. This suggestion gains support from the immunological identity of the high titer strain of RSV with its indigenous helper virus, RAV,[3] since the inability of RSV to synthesize its own coat would require that RAV direct coat synthesis for both viruses.

Two predictions, both of which are readily amenable to experimentation, arise from the concept that RSV is wholly dependent upon helper virus for production of the virus-specific portion of its coat. The first prediction is that NP cells contain no virus-specific coat antigens. NP cells should therefore be incapable either of absorbing RSV-neutralizing antibodies from antisera or of stimulating the synthesis of neutralizing antibodies when inoculated into chickens. The second prediction is that, if antigenically different helper viruses are employed, the antigenic structure of the virus-specific coat protein of RSV should be that of the helper virus used. Both predictions are explored in the experiments reported here and both are borne out by the results.

Material and Methods.—Terminology and notation: "Activation" refers to the process, mediated by helper virus, through which RSV becomes an infectious particle. "Virus-specific coat antigens" are defined here as those antigens found at the surface of a virus which are specified by a viral genome identical with, or related to, that enclosed by the coat. This term is intended to

distinguish the virus-specific antigens from other antigens which may be contributed to the virus coat by the host cell. The virus-specific antigens are the coat components which participate in the virus neutralization reaction, and they will sometimes be referred as to neutralizable antigens.

To distinguish RSV prepared from NP cells by activation with different helper viruses, the helper virus will be noted in parentheses after RSV. For example RSV (RAV) means RSV obtained from NP cells by activation with RAV.

RAV stock and assay: The preparation and assay of RAV stocks have been described.[3] The stocks contained about 1×10^8 infectious units of RAV per ml.

RIF stock and assay: RIF (resistance-inducing factor), which is a naturally occurring strain of avian visceral lymphomatosis virus originally obtained from the plasmas of congenitally infected chickens,[4] was purified by two terminal dilution passages in tissue culture. The final stock of RIF was prepared from chick embryo cells by the same technique previously described for preparation of RAV.[3] The infectivity of RIF was assayed by the comparative interference technique.[3] The stocks contained about 1×10^8 infectious units of RIF per ml.

RSV stock and assay: The high titer strain of RSV was used throughout this investigation. RSV(RAV) and RSV(RIF) were prepared from NP cells by adding 1×10^7 infectious units of RAV or RIF and harvesting the culture media daily for 9 days. The titer of RSV was assayed by the formation of Rous sarcoma foci on chick embryo cells. The titer of RSV was about 3×10^7 focus-forming units (FFU) per ml in the RSV(RAV) stock, and about 3×10^6 FFU per ml in the RSV(RIF) stock. RSV(RIF) was generally used as the challenge virus to detect the presence of interfering virus, since it was more readily inhibited than was RSV(RAV).[5] The ratio of RSV to helper virus in both RSV stocks was about 1:10.

NP cells: Secondary chick embryo cells were infected with an amount of RSV known to produce about 2 foci per plate. The infected cultures were overlaid with an 0.8% agar medium containing a 1:500 dilution of antiserum to RAV. At this dilution, the antiserum reduced the titer of RSV(RAV) to $<10^{-3}$ within 40 min. Seven days after infection fully developed Rous sarcoma foci were marked, and the agar was removed after being softened for 2 hr by the addition of 2 ml of culture medium. The cell sheet was washed with 4 ml of medium to remove floating cells, and 2 ml of solution containing 0.05% trypsin and a 1:500 dilution of antiserum to RAV were added. When the cells of the sheet had begun to round up prior to detachment from the plastic dish, the transformed cells in one focus were isolated in a glass capillary and added to a culture containing 10^6 normal chick embryo cells. These cultures were transferred serially at 3–4 day intervals, and the fluid medium was assayed for RSV at each transfer. A second and more sensitive test for virus production was usually carried out in which an aliquot of cells was X-irradiated and plated on normal chick embryo cultures. Under such conditions the X-rayed cells could not multiply to form a focus, but any virus released from them would initiate focus formation in the immediately adjacent normal cells.[1]

The media of cultures containing more than about 10^5 of the transformed cells was rapidly acidified, making it difficult to maintain such cultures for extended periods of time. It was therefore necessary from time to time to dilute the transformed cells upon transfer of the culture and to add them to a constant number (10^6) of normal chick embryo cells. The number of NP cells in mixed cultures was determined from the number of foci produced when the mixed cultures were diluted and plated on a background of normal chick embryo cells. The NP cultures used in most of the experiments contained about 2×10^5 transformed cells and 10^6 normal chick embryo cells per plate. A "line" of NP cells refers to those cells derived from a single Rous sarcoma focus.

Antiserum to RAV and its absorption with virus: Antiserum to RAV was obtained from chickens which had been infected intravenously with RAV 6–7 weeks earlier.[1] Absorption of antibody was carried out in the following manner. One-half ml of a dilution of the serum was mixed with 4.5 ml of a high concentration of the appropriate virus stock, and the mixture incubated at 37°C for 6 hr. It was then centrifuged at 30,000 rpm in the SW39 rotor of a Spinco ultracentrifuge for 30 min. The supernatant fluid was heated at 56°C for 30 min and used thereafter as antiserum in the neutralization test.

Neutralization test: One-tenth ml of the appropriate dilution of virus was mixed with 0.9 ml of each of a series of dilutions of heat-inactivated antiserum. After incubation at 37°C for 40 min, the surviving virus fraction of each mixture was assayed on chick embryo cultures.

Results.—Failure of NP cells to absorb antibody to RSV: Previous experiments have shown that neutralizing antibodies to RSV can be completely removed from an antiserum prepared against either RSV or RAV by stocks of either RSV or RAV.[2, 3] If virus-specific coat antigens existed in NP cells in amounts approaching those found in virus-producing Rous sarcoma cells, the antigens should be detectable by their ability to absorb RSV-neutralizing antibody. In an attempt to detect such antigens, two lines of NP cultures were prepared in 100 mm Petri plates, and 6 cultures of each line were superinfected with RAV to activate RSV; the remaining cultures were kept as controls. At 24 hr there was extensive production of RSV in the cultures superinfected with RAV, and no virus production in the controls. Both sets of cultures were washed, and a total of about 6×10^7 cells in each set was harvested in 5 ml by scraping the cells off the dish with a rubber policeman. The cells were disrupted in a glass homogenizer or in a sonic oscillator. The cell debris was removed by centrifugation at 3000 rpm for 5 min, and the supernatant fluid was collected. In a preliminary experiment, the supernatant fluids were added to diluted antisera prepared against RAV to determine whether RSV-neutralizing antibodies could be removed. The extracts obtained from the two lines of NP cultures failed to remove detectable amounts of antibody to RSV, while extracts obtained from the RAV superinfected cultures removed the antibody completely.

The result was inconclusive, however, because only about one fifth of the cells in the nonvirus-producing cultures were transformed and thereby potentially capable of producing viral antigen. The remaining cells were normal cells added to facilitate the maintenance and growth of the NP cells. By contrast, all the cells in the cultures infected with high concentrations of RAV could be presumed to be actively producing viral antigen. The failure to demonstrate antibody absorption by NP cultures would only be meaningful if it could be shown that these cultures had less than one fifth the antibody-absorbing capacity of the virus-producing cultures. To assay the concentration of neutralizable antigen in the virus-producing cultures, dilutions of a sonicated extract of the cells were used in the antibody absorption test. An undiluted sonicated preparation of NP cells was tested at the same time for its antibody absorbing capacity. The results are presented in Figure 1. It can be seen that a 1:10 dilution of the extracts from virus-producing cultures completely absorbed anti-RSV neutralizing antibody from the antiserum, and a 1:30 dilution absorbed almost half the antibody. The undiluted extracts from virus-free NP cells failed to absorb detectable quantities of antibody. These results indicate that NP cells, if they contain any virus-specific coat antigen at all, contain less than one sixth the amount found in virus-producing cells.

Production of tumors in chickens with NP cells, and absence of antiviral response: Experiments were carried out to determine whether NP cells which had arisen from *in vitro* infection of chick embryo cells by RSV could (*a*) produce tumors when inoculated into chickens, or (*b*) stimulate an immune response detectable either by the production of neutralizing antibody to RSV or the establishment of resistance in the chicken to later infection by RSV. In the first of two experiments, small numbers of NP cells were inoculated into the wing webs of two-week-old chickens, and the progress of tumor growth observed daily. The chickens were bled repeatedly up to the 50th day after inoculation, and the sera were tested for neu-

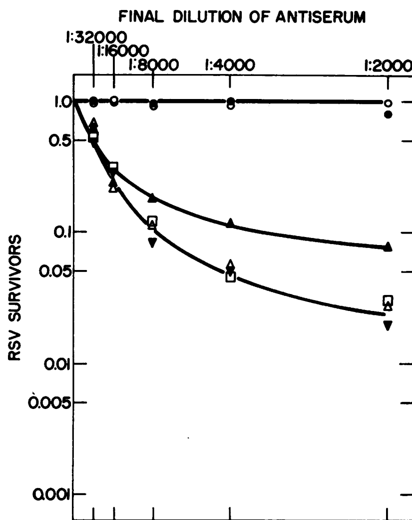

FIG. 1.—Failure to absorb antibody to RSV by extracts of NP cells. Extracts were made from NP cultures to which RAV had been added and from NP cultures to which no RAV had been added. (Each group of cultures contained a total of about 1.2×10^7 NP cells and 4.8×10^7 normal chick cells.) One-half ml of 1:20 dilution of antiserum to RAV was mixed with 4.5 ml of various dilutions of the extracts and incubated at 37° C for 6 hr. After removal of virus by centrifugation and heat treatment, antibody activity was measured by mixing various dilutions of the absorbed serum with RSV at 37° C for 40 min.

Antiserum absorbed with: complete medium (▼—▼); extract from NP cultures (not superinfected with RAV), undiluted (□—□); extract from RAV-superinfected NP cultures, undiluted (O—O), diluted to 1:10 (●—●), 1:30 (▲—▲), and 1:100 (△—△).

tralizing antibody to RSV. Some of the larger tumors were biopsied and tested for ability to produce RSV in tissue culture with and without the addition of RAV. At 57 days after inoculation, several chickens which had borne NP tumors were challenged with RSV to determine whether immunity to virus infection had been conferred by the NP cells.

In the second experiment, a similar schedule was followed, but two groups of chickens were included in addition to the group receiving NP cells. One of the additional groups received 10^4 NP cells together with 10^6 infectious units of RAV, to observe the response of chickens to inoculation with RSV-producing tumor cells. The other additional group was inoculated with 10^6 infectious units of RAV, to observe the response of nontumor-bearing chickens to viral antigen alone. Tests were made for neutralizing antibody and ability to withstand challenge infection with RSV.

The results of these experiments may be summarized as follows. It was found that inoculation of 2.7×10^2 or 2.7×10^3 NP cells into a chicken induced visible tumors within 5 days. The tumors continued to grow until about the 12th day after inoculation and then began to regress. In many cases the tumors had regressed by the 16th day after inoculation, but some of the regressed tumors reappeared within a week and grew progressively, eventually causing the death of the chicken. Autopsy of fatal cases revealed frequent metastases in the lung and occasional metastases in the kidneys. Six tumors were biopsied, and each tumor yielded cells which grew in tissue culture exhibiting the characteristic Rous sarcoma cell morphology. No tumor produced virus spontaneously, but every tumor retained the capacity for RSV activation by RAV.

The results of the tests for neutralizing antibody and susceptibility to challenge infection with RSV are presented in Table 1, experiments *a* and *b*. It can be seen that all chickens receiving NP cells developed tumors, but neutralizing antibodies to RSV could not be detected at the highest concentration of serum (1:10) tested. This is in marked contrast to the serum of chickens infected with RAV alone, since such chickens developed neutralizing antibodies to RSV detectable in all cases

TABLE 1

ABSENCE OF VIRUS-SPECIFIC AND TUMOR-SPECIFIC IMMUNOLOGICAL RESPONSE IN CHICKENS BEARING TUMORS INDUCED WITH NP CELLS

Expt. a

Initial inoculum	Inoculated sites developing tumors	Chickens developing tumors	Chickens with Antibody at 50 Days: Serum dilution 1:10	1:100	Latent Period (days)* for Tumor Development after Challenge with RSV at 57 Days: 10^2 FFU Individual sites	Mean	10^3 FFU Individual sites	Mean	10^4 FFU Individual sites	Mean
2.7 × 10^2 NP cells†	6/8	4/4	0/4	N.D.	10		7		7	
					11, 11	10	6, 7	6.5	6, 7	6.4
2.7 × 10^3 NP cells†	8/8	4/4	0/4	N.D.	(−) 9		6, 7		6, 6	
Nothing	...	...	0/3	N.D.	9, 10	9.8	7, 7	7.7	6, 6	6.8
					9, 12		8, 7		7, 7	
					9, 10		7, 9		7, 8	

Expt. b

Initial inoculum	Inoculated sites developing tumors	Chickens developing tumors	Chickens with Antibody at 40 Days: Serum dilution 1:10	1:100	Latent Period (days)* for Tumor Development after Challenge with RSV at 43 Days: 10^2 FFU Individual sites	Mean	10^3 FFU Individual sites	Mean
					10		10	
10^4 NP cells‡	21/21	15/15	0/15	0/15	9		8	
					8	9.25	8	8.8
					10		9	
10^4 NP cells‡ + 10^6 infectious units of RAV	16/16	16/16	§		§			
10^6 infectious units of RAV	0/20	0/20	20/20	20/20	No tumors developed in 4 infected chickens			
Nothing	...	...	0/7	0/7	9	8.75	8	8.0
					8		8	
					9		8	
					9		8	

N.D. = not done.

* Latent periods in the same horizontal row are from different sites inoculated with RSV in the same chicken.

† 0.1 ml of a suspension containing 2.7 × 10^2 or 2.7 × 10^3 NP cells plus 1.8 × 10^4 or 1.8 × 10^5 normal chick embryo cells, respectively, was inoculated.

‡ 0.1 ml of a suspension containing 1 × 10^4 NP cells plus 1 × 10^6 normal chick embryo cells was inoculated.

§ All chickens dead within 29 days after inoculation of NP cells plus RAV.

at a 1:100 dilution of serum. All chickens which received RSV-producing cells died with massive tumors 29 days after inoculation, so that their sera could not be included in the immunological tests.

The parameters used to measure susceptibility of the chickens to challenge infection with RSV were the incidence of tumors induced by inoculation with various dilutions of RSV, and the latent period for tumor development. As can be seen in Table 1, experiments *a* and *b*, the chickens which had supported the growth of tumors induced by NP cells were as susceptible to challenge infection with RSV as were control chickens which had had no experience with NP cells. This conclusion is based on the development of tumors after RSV infection at every site, except one, in the 7 chickens which had borne tumors induced by NP cells, and on the lack of a significant difference in latent periods for tumor development between these chickens and the control chickens which had had no prior inoculation. The lack of immunity to RSV in chickens bearing NP tumors is in sharp contrast to the high degree of immunity to RSV developed by the chickens as a result of infection with RAV (Table 1, experiment *b*). Not a single tumor developed in this group even when 10^3 FFU of RSV were inoculated. The failure of NP cells to

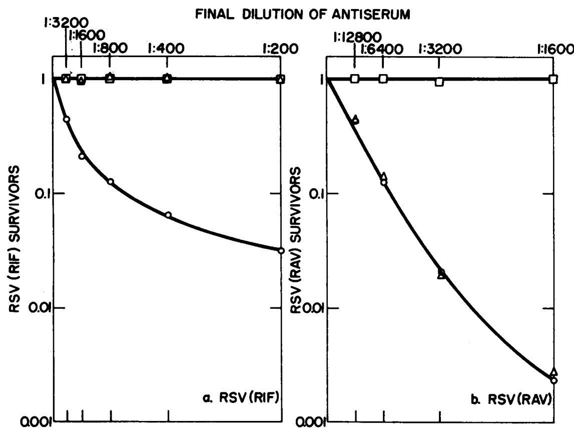

FIG. 2.—Neutralization of RSV(RAV) and RSV(RIF). Antiserum to RAV was diluted to 1:20. One-half ml of the diluted serum was mixed with 4.5 ml of fluid containing 4.5 × 10^8 infectious units of RAV and RIF, respectively, and incubated at 37° C for 6 hr. After removal of virus by centrifugation and heat treatment, antibody activity was measured by mixing various dilutions of the absorbed serum with RSV(RIF) and RSV(RAV), respectively, at 37° C for 40 min. Fig. 2*a* represents neutralization of RSV(RIF), and 2*b*, neutralization of RSV(RAV).

Antiserum absorbed with: complete medium (O—O), RAV (□—□), and RIF (Δ—Δ).

engender immunity in chickens to infection with RSV is consistent with the failure of these cells to induce the production of neutralizing antibodies to RSV. Both results lead to the conclusion that NP cells contain no RSV-specific coat antigens.

Specification of RSV antigenicity by helper virus: The second prediction arising from the idea that RSV does not specify its own protein coat is that RSV bears the antigenic structure of whatever helper virus is used for its activation. This prediction can be tested by use of two antigenically distinguishable helper viruses such as RIF and RAV appear to be.[2]

An experiment was carried out to verify the antigenic similarities and differences between RIF and RAV. Various samples of an antiserum prepared against RAV were absorbed with either RIF or RAV, and the absorbed sera, as well as the original unabsorbed serum, were tested for their ability to neutralize RIF and RAV. It can be seen from the results of this experiment, presented in Table 2, that the unabsorbed antiserum to RAV neutralizes both RIF and RAV, although it is less active against RIF than against RAV. Absorption of the antiserum with RIF removes all the neutralizing activity against RIF but leaves fully intact the neutralizing activity against RAV. In contrast to the restricted absorption by RIF, RAV removed all neutralizing activity against RIF, and about 90 per cent of neutralizing activity against RAV. (The extent of absorption of the anti-RAV activity by RAV was quantitated by comparing the results with a standard RAV-neutralization curve.) The use of absorbed sera therefore established a clear-cut antigenic difference between RIF and RAV.

TABLE 2

NEUTRALIZATION OF RIF, RAV, RSV(RIF) AND RSV(RAV) BY ANTISERUM TO RAV ABSORBED WITH RIF AND RAV

Antiserum to RAV absorbed with	Final dilution of serum	Surviving Fraction of Infectious Particles			
		RIF	RAV	RSV(RIF)	RSV(RAV)
Complete medium	1:200	0.06	<0.001	0.05	<0.001
(Unabsorbed)	1:800	0.37	<0.001	0.30	<0.001
RIF	1:200	1.0	<0.001	1.0	<0.001
	1:800	1.0	<0.001	1.0	<0.001
RAV	1:200	1.0	0.02	1.0	0.017
	1:800	1.0	0.55	1.0	0.50

Antiserum to RAV was absorbed by RIF and RAV as described in Fig. 2. One ml of each absorbed antiserum was then mixed with 0.1 ml of virus suspensions containing 10^7 infectious units of RIF or RAV, and 1 × 10^6 FFU of RSV(RIF) or RSV(RAV) respectively, and incubated at 37°C for 40 min. The surviving fraction of RSV(RIF) and RSV(RAV) was assayed by focus formation and the surviving fraction of RIF and RAV by the comparative interference technique.

As a supplement to the above experiment, the absorbed and unabsorbed antisera were also tested for their ability to neutralize RSV(RIF) and RSV(RAV). Comparison of the neutralization of RSV(RIF) and RSV(RAV) with the neutralization of RIF and RAV in Table 2 shows that RSV is inactivated to the same extent as its associated helper virus. Another cross-absorption of the antiserum to RAV was carried out in which the residual antibody titers against RSV(RIF) and RSV(RAV) were measured over a wider range of serum dilutions than was used previously. It can be seen from the results in Figure 2 that RIF absorbed all the neutralizing activity against RSV(RIF) and none against RSV(RAV), while RAV absorbed all the activity against both types of RSV. Therefore, RSV activated by different helpers was again shown to have the same sensitivity to inactivation by absorbed antisera as its respective helper.

Discussion.—The techniques used here to detect the putative presence of virus-specific coat antigen in NP cells differ in sensitivity. The failure of disrupted NP cells to absorb measurable amounts of RSV neutralizing antibody from a RAV antiserum merely sets an upper limit on the amount of RSV coat protein present in these cells. But the test of the ability of NP cells to induce an immune response in chickens is probably a much more sensitive indicator for the presence of viral antigen. While a reliable estimate cannot be made of the minimum immunogenic dose of RSV particles, information from other systems suggests that very small numbers of virus particles suffice to induce detectable neutralizing antibodies. For example, Uhr *et al.*[6] reported recently that as few as 6×10^4 bacteriophage particles induce the formation of neutralizing antibodies at a maximum initial rate in guinea pigs. The coat antigens of RSV are highly antigenic in the strain of chickens employed in the present investigation, as indicated by the regular production of sera with very high titers of neutralizing antibodies from chickens infected with RAV. Considering the opportunities for repeated antigenic stimulation in chickens with actively growing tumors, it seems likely that the production of very small amounts of RSV coat antigen by transplanted NP cells would induce the formation of neutralizing antibody. Nevertheless, no RSV neutralizing antibody could be detected after 50 days, when some of the tumors had reached a diameter of more than 3 cm, and probably contained more than 10^9 cells. Since it is unlikely that as many as 10^9 RSV particles are required to induce the formation of neutralizing antibody, it is concluded that there is less virus coat antigen in a single NP cell than that carried by a single mature virus particle.

The failure of chickens carrying NP tumors to resist RSV infection not only reinforces the conclusion of the absence of virus coat antigen in NP cells, but suggests that the NP cells have no unique *tumor* antigen equivalent to the transplantation antigen reported for tumors induced by polyoma virus.[7] If such an antigen were present, we might expect to find evidence for at least a partial resistance in chickens with NP cell tumors to tumor induction by RSV. Nevertheless, such chickens all developed tumors when infected with 100 FFU of RSV. Even the latent period for the appearance of the tumors was unaffected by the prior exposure to NP cells. Since latent period has been shown to be a reliable and sensitive indicator of the biologically effective dose of RSV in the dose range used here,[8] the results suggest that NP cells contain no tumor-specific transplantation-type antigen. (The early, partial regression of NP cell tumors is probably due

to normal histocompatibility differences between donor and recipient since the chickens were not highly inbred.)

That RSV bears the antigenic imprint of the particular helper-virus used in its activation is in harmony with the absence of the viral coat antigen from NP cells. While there is no established precedent in animal virology for the complete dependence of one virus on another virus for specification of its coat antigens, the presence of antigens from two related viruses in the coat of one of them is well known. This is the phenomenon of phenotypic mixing which is observed when two related, but antigenically distinguishable viruses are grown in the same cell. The phenomenon was first reported with bacteriophages,[9] and has been observed with the viruses of influenza[10] and Newcastle disease[11] which are similar in structure and composition to RSV. The proteins responsible for the antigenic specificity of phenotypically mixed viruses appear to be withdrawn from a pool at random to enclose individual virus genomes. In a mixedly infected cell, a high proportion of the virus particles which are formed have the combined antigenic specificity of both parents, but the genome of one or the other. In the case of a cell mixedly infected with RSV and a helper virus, the scheme has to be modified since *only* the helper virus can direct the synthesis of coat protein. Although both viruses have the antigenic specificity of the helper virus, the underlying mechanism of the random withdrawal of antigenic components by both genomes may be the same as that of phenotypic mixing.

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† The first paper of this series was subtitled, "I. Characterization of the helper virus." [3]

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