RESPONSE OF CELL AND ORGANISM TO INFECTION WITH AVIAN TUMOR VIRUSES[1]

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I. Introduction

Fortunately, there is no need for a lengthy preamble to justify the present discussion nor the research which occasions it. The significance of tumor virus research has become evident to all biologists as a result of the isolation of a number of agents from mouse neoplasms during the past decade. This recognition is somewhat belated as tumor viruses have been known since 1908 when Ellermann and Bang (6) isolated the agent responsible for erythroblastosis in chickens. There was at first considerable reluctance to admit the importance of this finding for cancer research, partly because the neoplastic nature of leukemia cells was questioned. This question became irrelevant a few years later after the isolation of the Rous sarcoma virus (RSV) from a naturally occurring connective tissue tumor in a hen (12). In subsequent years, viruses were isolated from a variety of chicken sarcomas and leukemias. All the viruses isolated from chicken tumors up to the present appear to be closely related to one another as indicated by similarities in size, morphology, chemical constitution, and antigenicity, and it is convenient to classify them together as agents of the avian leukosis complex (2). Agents of this complex cover a wide spectrum of virulence; RSV has been so effectively adapted for rapid growth in the laboratory that it invariably produces highly malignant tumors within a few days, whereas visceral lymphomatosis virus (VLV) does not induce malignancy for many months and even then with irregularity. Other leukosis viruses such as the myeloblastosis and erythroblastosis viruses have intermediate degrees of virulence.

Under natural conditions VLV is by far the most widely occurring of the leukosis viruses as indicated by the fact that there is not a flock of chickens in the United States known to be free of the associated disease condition called lymphomatosis. In many respects the pathogenesis of the lymphomatosis in chickens resembles the pathogenesis of lymphocytic leukemias in higher organisms. Therefore, if one were to choose an agent as a model to understand the general patterns of viral carcinogenesis, VLV would be the natural choice. Unfortunately, until recently the assay system for this virus has been too cumbersome to permit integrated experimental investigation of its behavior.

In lieu of an efficient assay system for VLV, it has been necessary for virologists to turn to other systems such as RSV as possible models for investigation of the detailed interactions between tumor viruses and cells. In the case of RSV an efficient and precise assay system in vitro was developed (20) and rapid progress made in understanding the interactions between virus and cell which lead to malignancy. During the course of the work with RSV, an assay was inadvertently discovered for VLV. Although this assay does not have all the desirable features of the assay for RSV, it has proved to be a useful tool for studying certain aspects of tumor virus infection which cannot be investigated with RSV, namely, the roles played by congenital transmission and immunological tolerance in per-

[1] Text of the Eli Lilly and Company Research Award Address in Bacteriology and Immunology presented at the Annual Meeting of the American Society for Microbiology in Chicago, Ill., on April 25, 1961. Based on research carried out under the U. S. Public Health Service grant, C-4-774.

petuating virus in nature and causing disease. Therefore, this paper will be divided into two relatively independent sections, one concerned with RSV infection at the level of the cell and the other concerned with VLV infection at the level of the organism.

II. Rous Sarcoma Virus Infection at the Cellular Level

In describing RSV infection at the level of the cell, I shall confine myself to recent kinetic and cytological studies carried out in vitro (14, 21, 23). When a high concentration of RSV is added to chick embryo cells growing in tissue culture the cells undergo a series of characteristic morphological alterations. At about 2 days after infection, the fibroblasts, which are normally fusiform in shape, become plumper and their refractility increases. Within the next day or two, the cells become more rounded and escape from contact inhibition. Escape from the contact inhibition which restricts normal fibroblasts to monolayer growth permits the Rous sarcoma cells to move freely over one another and over normal cells. As a result, Rous sarcoma cells are frequently found in several layers. If only a small number of RSV infectious units are added to a chick embryo culture, colonies of Rous sarcoma cells (foci) become visible against a background of normal cells (Fig. 1 and 2) in 5 to 6 days. Rous sarcoma cells can readily be distinguished from normal chick embryo fibroblasts because of the rounded morphology of individual cells and the multilayered growth of the colony.

The morphological changes in tissue culture occur at the same time and are of the same type as those which occur in vivo (10). The growth of virus also follows the same pattern in vitro and in vivo (4, 11, 13). Therefore, it is likely that the cellular events studied in tissue culture are an accurate reflection of the events leading to malignancy in the animal.

The number of foci produced in a culture varies linearly with the concentration of RSV added (20). Up to 1,000 such foci can be counted on a single 50-mm petri dish. Focus formation therefore serves as an excellent assay for virus infectivity.

Experiments on the growth of Rous sarcoma virus in vitro revealed the following features (Fig. 3). There is an eclipse period of about 12 hrs after infection during which very little virus

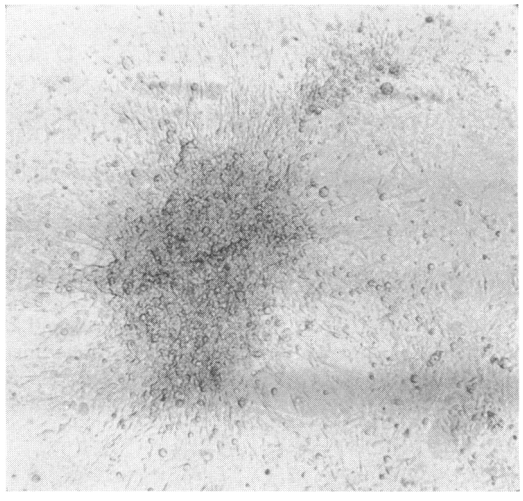

FIG. 1. *Low power view of a typical RSV focus at 7 days on a culture of chick embryo cells. This figure first appeared in Virology* ***12:14–31****, 1960. See reference (14).*

can be recovered from the infected cells. Thereafter, progeny virus particles appear and their number increases exponentially until about 3 days after infection. At this time the cells reach a constant rate of virus production of about 1 infectious unit of virus per cell per hr (23). Since the ratio of virus particles to infectious units has recently been estimated by Crawford (5) as about 1,000, it would appear that an infected cell releases about 1,000 virus particles per hr. The volume of the virus being about 10^{-6} that of the cell, the cell must produce about $\frac{1}{1000}$ its own mass in virus per hr. The growth rate of the cells is apparently unaffected by this demand on its synthetic activity.

A striking aspect of the growth of RSV is its rapid and continuous release from the infected cell. This was first suggested by the observation that virus can be detected in the medium before it can be detected in association with washed cells when samples are taken at time intervals as short as 4 hr, and that the level of virus in the medium usually exceeds that in the cells by a factor of about 10 during the first few days of virus production (21, 23). This relationship between cell-associated virus and free virus can only occur if there is a rapid and continuous release from the cell of mature virus particles as they are completed (17).

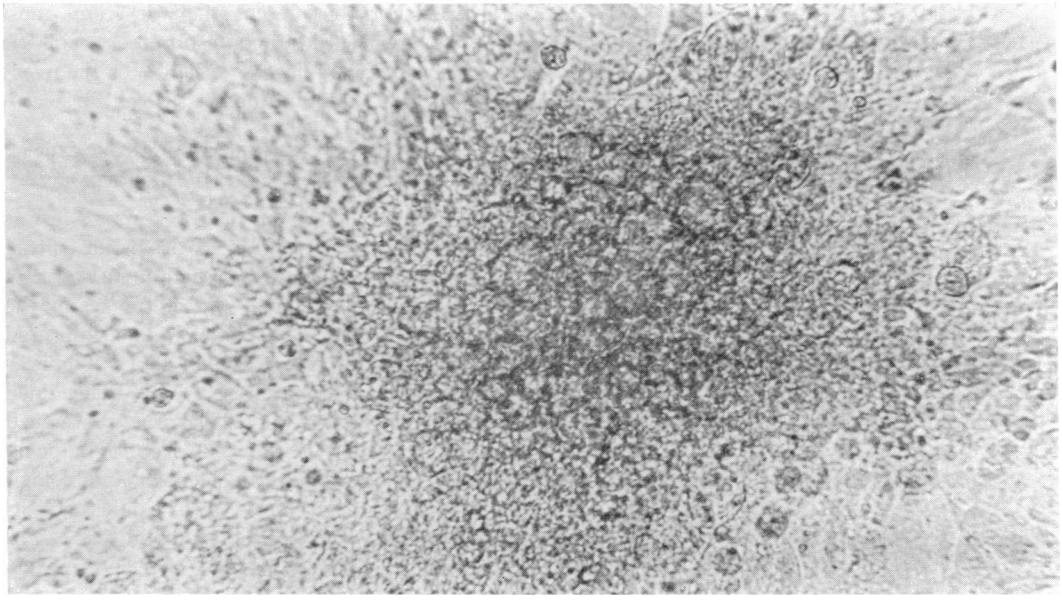

FIG. 2. *Higher magnification of RSV focus in Fig. 1*

The rapid and continuous release of virus from cells is a common feature of the multiplication of large RNA viruses, a category which includes influenza, mumps, and Newcastle disease virus in addition to the avian tumor viruses (16). A variety of findings have accrued over the years to indicate that there is an association between rapid, continuous release of a virus and its maturation at the cell membrane. Further support for the completion of RSV at the cell surface came from the finding that over 90% of the virus associated with washed, intact cells was accessible to inactivation by antiserum (23). Since the antibody could not penetrate the intact cell, it may be assumed that almost all the cell-associated virus is superficial, at least during the early stages of virus growth.

Since the surface area of the virus is about 10^{-4} that of the cell, the release of 1,000 particles per hr is equivalent to the loss of $\frac{1}{10}$ the surface area of the cell every hour. It is not unreasonable, therefore, to assume that virus multiplication may interfere with the function of the cell membrane. This will be discussed below at greater length.

A major deficiency of the kinetic experiments on infected cells is that they give no hint of the amount of viral protein which is not in mature virus particles. Thus, the calculated ratio of virus surface to cell surface is likely to be underestimated. The kinetic experiments also lack the impact of direct visualization of virus growth. It was with these deficiencies in mind that an analysis was undertaken of RSV growth and localization with the fluorescent antibody technique (23). The general plan of this work was to infect chick embryo cultures with RSV and stain individual cultures with fluorescent antibody at daily intervals. The resulting observations are described below.

The first appearance of viral antigen can be detected along the borders of infected cells at 2 days after infection (Fig. 4). As previously noted it is at this time that the first morphological signs of virus infection become apparent, and it is also at this time that the production of virus in a significant fraction of infected cells can be detected. Proof that the viral antigen is indeed at the cell surface arises from the fact that living cells can be stained with fluorescent antibody just as effectively as fixed cells (Fig. 5).

At 3 to 4 days, a marked change in cell behavior occurs. The cells become more rounded in outline and escape contact inhibition. The escape from contact inhibition permits the altered cells to move over one another and over normal cells. In this respect they assume the characteristic behavior described by Abercrombie,

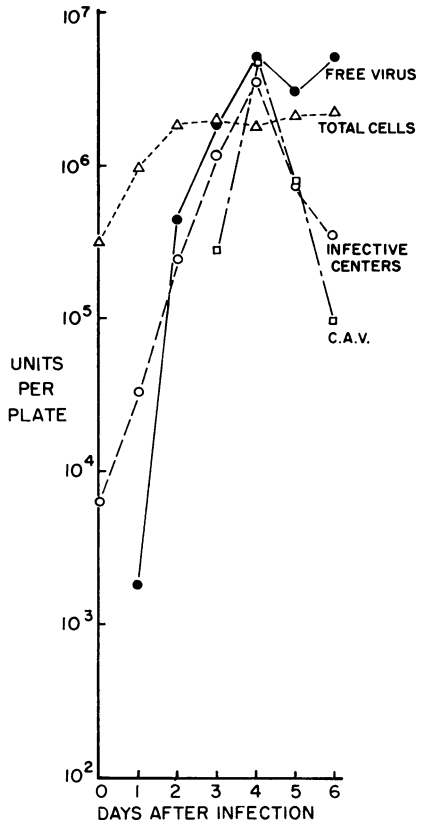

FIG. 3. *RSV multiplication in cultures of chick embryo cells. This figure first appeared in Virology 12:1431, 1960. See reference (14). C.A.V. = Cell-associated virus.*

Heaysman, and Karthauser (1) for sarcoma cells. When cells of this stage are stained with fluorescent antibody, it is found that large amounts of viral antigen are being shed from the cell surface into a matrix-like substance around the cell (Fig. 6). It seems likely that the contemporaneous occurrence of virus shedding at the cell membrane and the loss of contact inhibition is more than coincidental since the cell membrane is believed to be the site at which contact inhibition is mediated (1).

Following the shedding stage, the synthesis of viral protein can be detected both in the cytoplasm and at the cell surface (Fig. 7). Since the appearance of cytoplasmic antigen occurs after the cell has escaped contact inhibition, it is unlikely that production of virus within the cytoplasm plays a central role in this primary change of cell behavior though it might play some role in perpetuating the malignant behavior of the cell. For the present then, it seems most likely that the earliest manifestation of neoplastic behavior in a cell infected with RSV is the result of virus-induced alterations at the cell surface.

III. Resistance-Inducing Factor and Lymphomatosis Virus Infection

During the course of the RSV work, it was found that the cells obtained for tissue culture from certain embryos were highly resistant to RSV infection. Although the cultures and the embryos from which they were obtained appeared normal, a virus was isolated from the embryos which could induce resistance to RSV when added to sensitive cultures (15). The virus was named RIF, an acronym for "resistance-inducing factor," but in its physical, chemical, and biological characteristics RIF proved to be indistinguishable from VLV (7). It was also found that established strains of VLV could be detected in vitro by interference with RSV with precisely the same technique used to assay RIF. These facts plus epidemiological observations to be discussed below indicate that RIF is a strain of lymphomatosis virus (3).

It was found that about 1 in 40 embryos from an ordinary flock of chickens was congenitally infected with RIF. However, the frequency of infected embryos from an experimental flock which had been selected for a high incidence of lymphomatosis was about 1 in 4 (18). The lymphomatosis-susceptible flock was made available to us (courtesy of Kimber Farms, Niles, Calif.) for a study of the congenital transmission of RIF.

The titer of RIF in an unknown preparation was determined by infecting RSV-sensitive cultures with serial dilutions of the sample and challenging aliquots of cells from the cultures with RSV at each of three or four successive cell transfers. A high concentration of RIF induced resistance to RSV at the first transfer, whereas lower concentrations induced resistance at subsequent transfers. By reference to standard curves for a preparation of known infectivity, the titer of the unknown could be determined.

Antibody to RIF could be determined by its ability to eliminate the RSV-inhibitory activity of RIF. However, a large-scale study of the distribution of virus and antibody was under consideration, and the RIF neutralization was too cumbersome to be used on such a scale. It could be shown that the level of neutralizing activity of a serum against RSV was a good indicator of the level of its activity against RIF. Since the

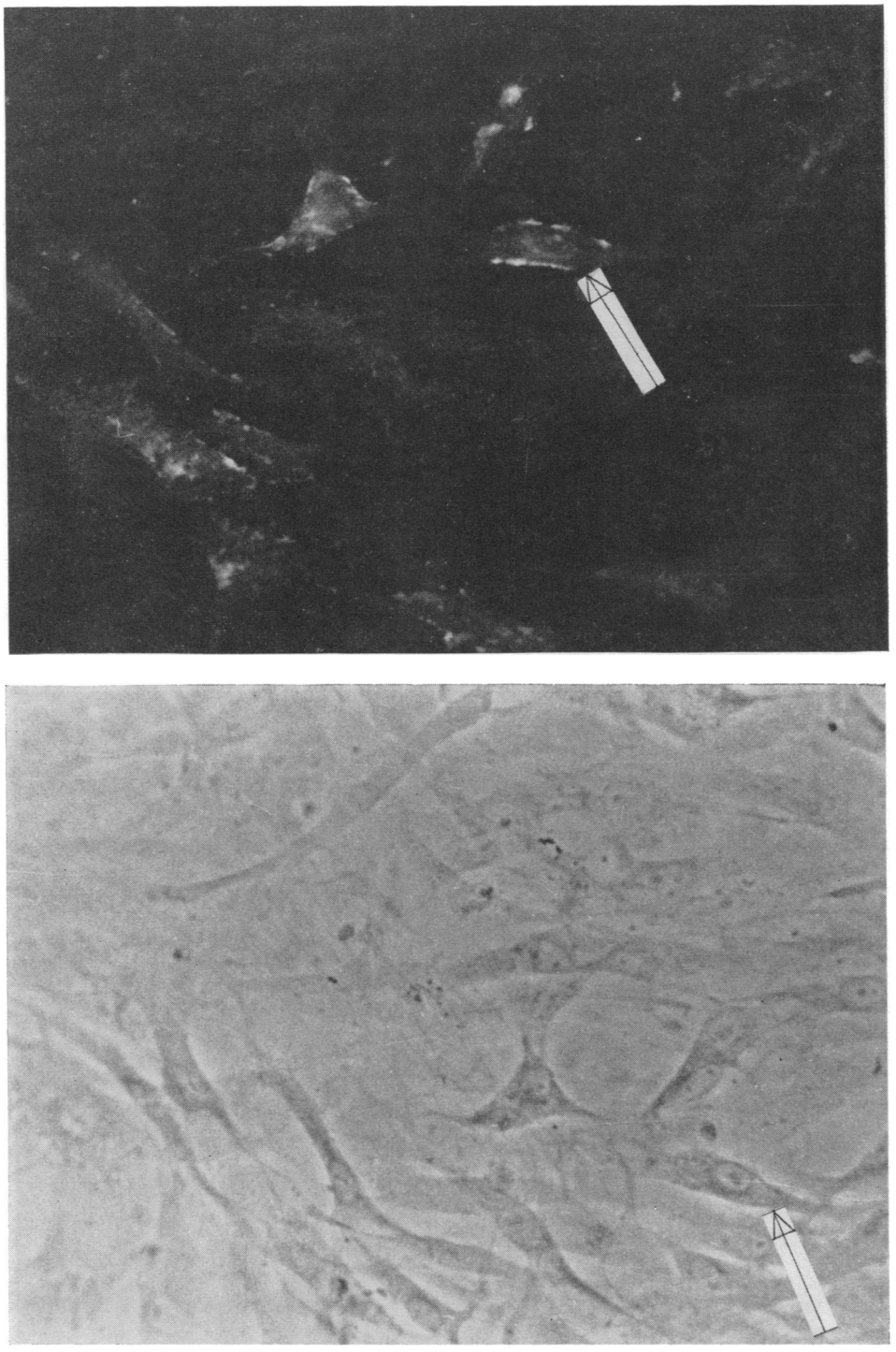

FIG. 4a (*top*). *Chick fibroblast culture, fixed and stained with fluorescent RSV antiserum on the 3rd day after infection. Viral antigen (arrow) appears at the cell membrane. Scale: 2.9 cm = 100 μ.*

FIG. 4b (*bottom*). *Same preparation under phase contrast. This figure first appeared in Virology* **13:** *528–544, 1961. See reference (23). Scale: 2.9 cm = 100 μ.*

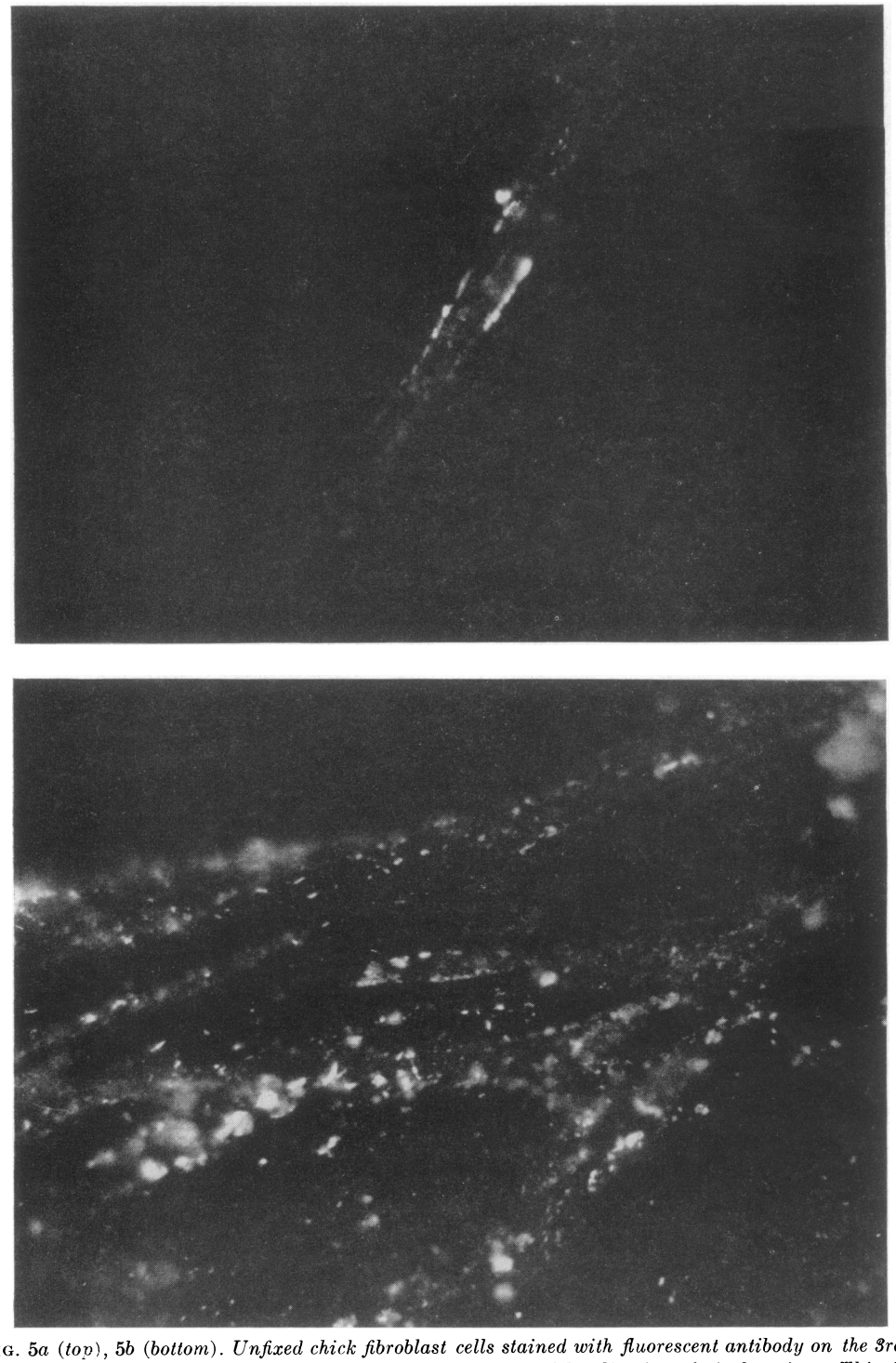

FIG. 5*a* (*top*), 5*b* (*bottom*). *Unfixed chick fibroblast cells stained with fluorescent antibody on the 3rd* (*a*) *and 5th* (*b*) *day after infection, respectively, showing superficial localization of viral antigen. This figure first appeared in Virology* **13:** *528–544, 1961. See reference* (*23*). *Scale:* (*a*) *5.3 cm = 100 μ;* (*b*) *2.3 cm = 100 μ.*

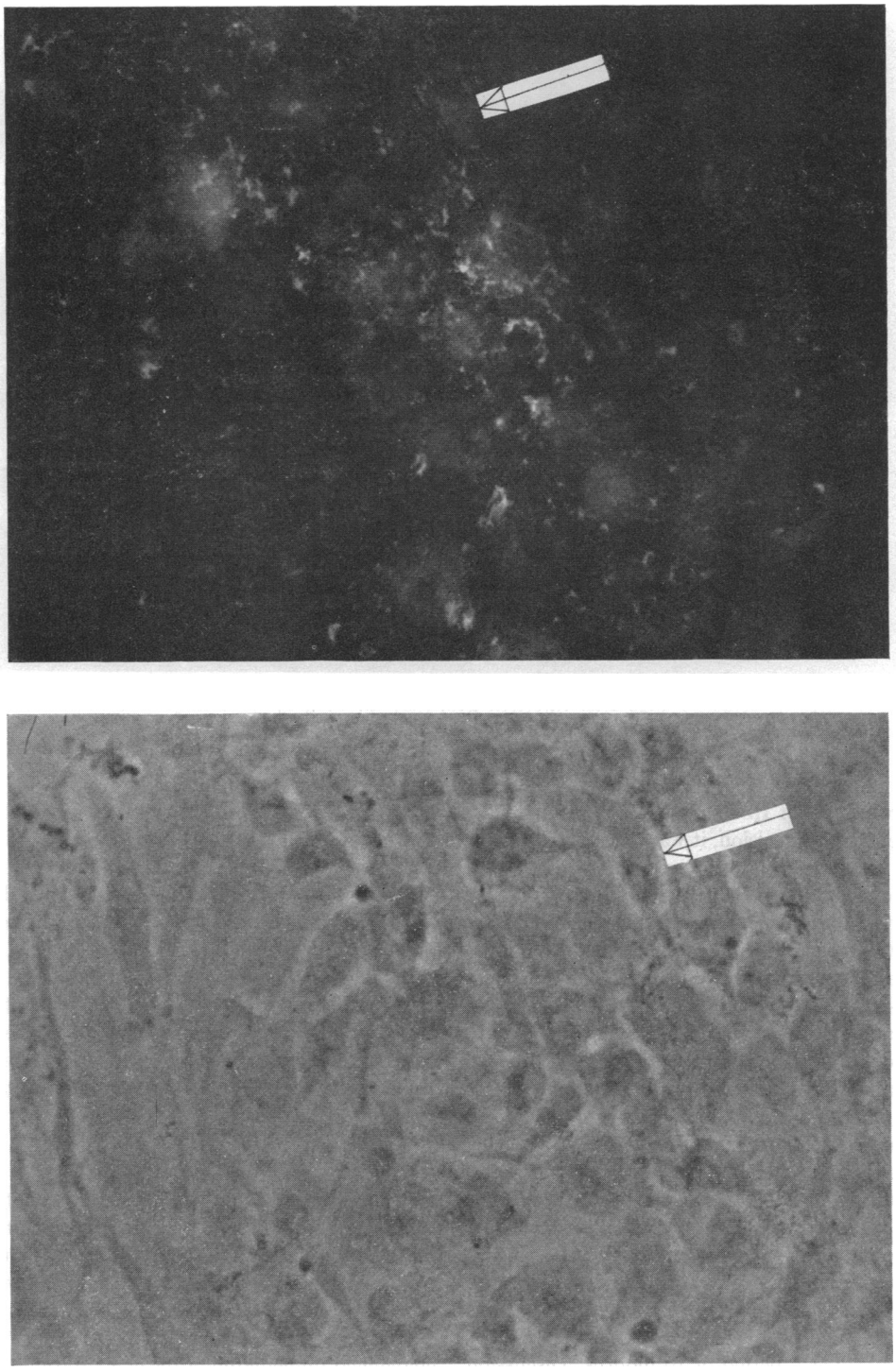

FIG. 6a (*top*), 6b (*bottom*). *A focus of Rous sarcoma cells stained with fluorescent antibody and seen in fluorescent* (a) *and phase contrast* (b) *microscopy. Arrow points to same cell in both photographs. Viral antigen is being shed from cell surface. Scale: 3 cm = 100 μ.*

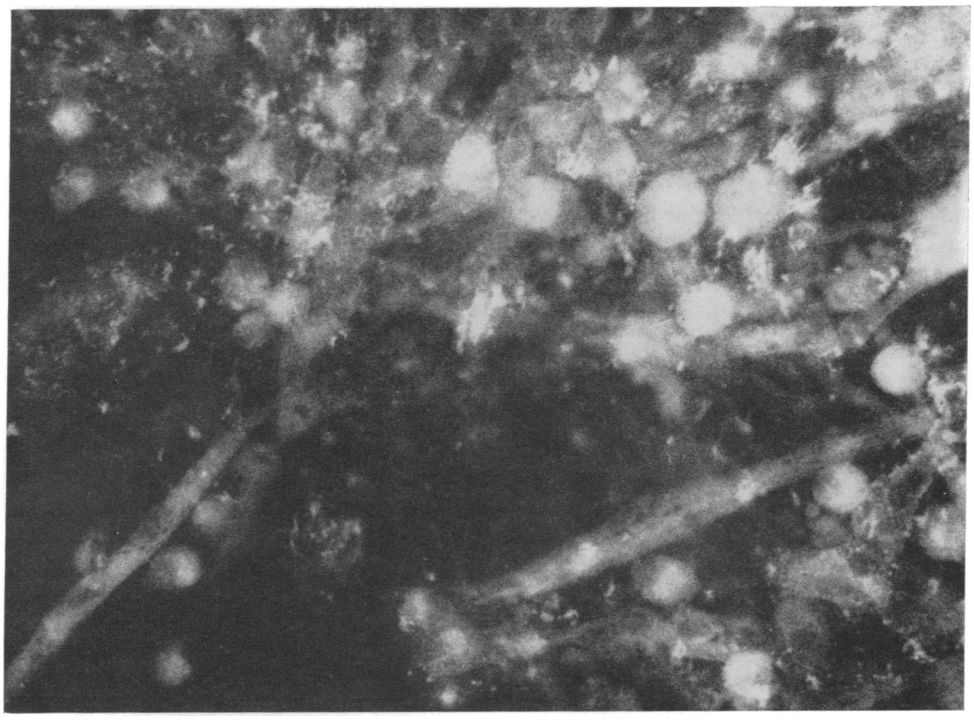

FIG. 7. *Chick fibroblasts 6 days after infection with RSV showing cytoplasmic fluorescence. This figure first appeared in Virology 13: 528–544, 1961. See reference (23). Scale: 2.3 cm = 100 μ.*

neutralization of RSV could be carried out with efficiency and precision, it was substituted for the RIF neutralization to assay antibodies to RIF in the population. Subsequent tests of selected sera for ability to neutralize RIF showed that the RSV neutralization gave an accurate picture of the distribution of RIF-neutralizing antibody.

The plan of the study mentioned above was to determine the status of the parental birds with regard to viremia, antibody, and ability to transmit RIF congenitally. The parental birds were bled repeatedly, and embryos of known parentage were obtained by trap-nesting hens. The embryos were used to prepare cultures and the cultures were challenged with RSV to determine whether they were infected with RIF.

The titers of RIF and antibody in the blood of each parental bird were determined once during the egg-laying period when the parents were 12 months old and at three subsequent intervals over a period of 10 months. Discussion of the results is simplified by the fact that there was little change in the occurrence and titers of virus and antibody in individual birds during this period. The adults could be divided into two classes consisting of viremic birds and nonviremic birds (Table 1). Four of the 18 hens in the initial study and 3 of the 8 roosters had high titers of virus present in the blood throughout the 10-month period of study. None of the viremic birds had antibodies to RIF and only 1 of the 8 had antibodies to RSV. It is evident that persistent viremia and neutralizing antibodies are to a large extent mutually exclusive, suggesting that the viremic birds were immunologically tolerant to the virus.

A. Congenital Transmission of RIF

There was a marked distinction between viremic and nonviremic birds in ability to transmit RIF to progeny. All the fertile viremic females were persistent congenital transmitters of the virus (Fig. 8). Most of the cultures from these embryos were highly resistant to RSV when challenged immediately after explantation of the cells, indicating that a high proportion of cells had been actively producing virus in ovo. Only a few showed the delayed resistance which is

TABLE 1.[a] *RIF-viremia and antibody in parental birds*

	Viremic birds[b]			Nonviremic birds[c]	
	Antibody to RSV[d]	Antibody to RIF[e]		Antibody to RSV[d]	Antibody to RIF[e]
Hen no.:			*Hen no.:*		
1	−	−	2	−	+
3	−	−	4	+	+
7	−	−	5	+	+
9	−	−	8	−	+
Rooster no.:					
R1	+	−	10	+	+
			11	+	+
R2	−	−	12	+	+
R9	−	−	13	+	+
			14	−	+
			16	−	+
			17	+	+
			18	+	+
			19	+	+
			20	+	+
			Rooster no.:		
			R3	+	+
			R4	+	+
			R6	+	+
			R8	+	+
			R10	+	+

[a] This table first appeared in Proc. Natl. Acad. Sci. U. S. **47**:1058–1060, 1961. *See* reference (18).

[b] Viremic birds = serum-induced resistance to RSV in the first transfer when obtained at 12, 14, 17, and 22 months of age and added to RSV-sensitive cultures.

[c] Nonviremic birds = serum failed to induce resistance in cultures challenged with RSV in three successive transfers.

[d] Antibody to RSV. (+) = A 1:10 dilution of serum-reduced RSV titer ≥ 10-fold in 40 min of incubation at 37 C. (−) = A 1:10 dilution of serum-reduced RSV titers ≥ 2-fold in 40 min of incubation at 37 C.

[e] Antibody to RIF. (+) = A 1:10 dilution of serum eliminated the RSV-inhibitory effect of a 1:10 dilution of RIF in cultures challenged with RSV after 1 transfer. (−) = A 1:10 dilution of serum failed to eliminate the RSV-inhibitory effect of RIF.

characteristic of those embryos in which only a small proportion of cells is infected at the time of explantation.

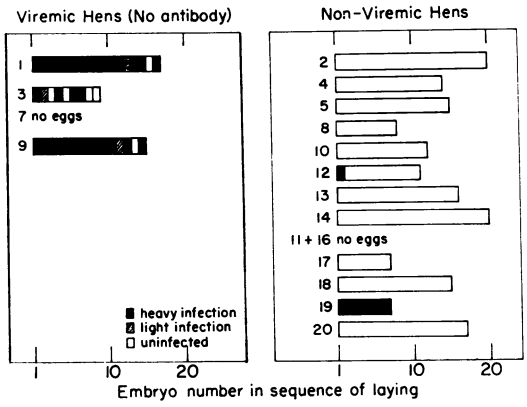

FIG. 8. *Congenital transmission of RIF. The chart shows the embryos from viremic and nonviremic hens in the order that the egg was laid. This figure first appeared in Proc. Natl. Acad. Sci. U. S. 47:1058–1060, 1961. See reference (18).*

Among the nonviremic females, only one was a persistent congenital transmitter. This one is of special interest, however, since its existence shows that virus multiplication can continue indefinitely in an animal despite the continuous presence of antibody. This finding has more recently been substantiated in studies on a larger number of birds of this flock in which about 1 out of every 7 nonviremic hens was found to be a persistent congenital transmitter despite the presence of antibody. This finding brings up the question whether RIF continues to multiply in *non*ovarian tissue in the remaining nonviremic birds, i.e., those which failed to congenitally transmit the virus. If so, the persisting antigenic stimulus would provide a simple explanation for the constancy of antibody titer over many months.

There was no suggestion of congenital transmission by viremic males (Table 2). Of the 4 nonviremic females mated to viremic males, 3 produced 37 progeny, all of which were uninfected. The remaining hen, no. 19, which produced infected progeny, continued to do so when mated to a nonviremic male. These results, which have been substantiated in larger numbers of birds, indicate that congenital transmission was under strict maternal control. Similar results have been reported for mouse leukemia virus (8).

One possible explanation for the failure of male transmission is the likelihood that any mature virus which might be carried in the sperm fluid would be inactivated upon contact with antibody in the female. When females with low antibody

TABLE 2.[a] *Failure of congenital transmission of RIF by viremic roosters*

Viremic roosters				Nonviremic roosters			
Rooster no.	×	Hen no.	Infected progeny/ total progeny	Rooster no.	×	Hen no.	Infected progeny/ total progeny
R1	×	18	0/15	R3	×	14	0/20
R2	×	17	0/7	R4	×	13	0/16
R9	×	19[b]	7/7	R6	×	4	0/14
R9	×	20	0/17	R6	×	5	0/15
				R8	×	1[c]	16/17
Total.....			7/46	R8	×	2	0/20
				R8	×	8	0/8
				R8	×	9[c]	14/15
				R8	×	10	0/12
				R8	×	12	1/11
				R10	×	3[c]	5/9
				Total.....			36/157

[a] This table first appeared in Proc. Natl. Acad. Sci. U. S. **47**:1058-1060, 1961. *See* reference (18).

[b] Continued to produce infected progeny when mated with nonviremic rooster.

[c] Viremic hens.

titers were mated to viremic males, however, infected progeny were not produced (18). Unfortunately, antibody-free females were not available, and an unequivocal result could not be obtained. The most revealing aspect of the failure of male transmission is its implication for the localization of the viral genome in the cell and in this respect it seems unlikely that antibody would affect the transmission of the viral genome particularly if carried within spermatozoa.

Another possible explanation for the failure of male transmission is that the testicular cells of the viremic male had somehow escaped infection. This possibility could be explored by trypsinizing, washing, and suspending the testicular cells and plating them as infective centers. When this procedure was carried out, it was found that a high proportion of cells obtained from the testes of viremic males were actively producing virus (18).

The cells obtained by this technique presumably were the larger and less differentiated elements of the testes since spermatozoa were not seen. The failure of the male transmission, therefore, suggests that the viral genome is lost from the cell during the process of spermiogenesis, when RNA and cytoplasm are shed from spermatocytes to form spermatozoa. This experiment, then, indicates that, unlike the temperate bacteriophages, the genome of an RNA virus is not closely associated with chromosomes of the cell.

B. The Fate of Congenitally Infected and Uninfected Chicks

As noted above, the high resistance to RSV of cells from congenitally infected embryos immediately after explantation suggested that a high proportion of cells in the embryo was infected. This seemed remarkable in view of the fact that the infected embryos appeared to be normal in every way and indeed usually hatched and matured into normal adult chickens. To seek confirmation of the impression that a high proportion of cells from the embryo was infected, the cells were plated as infective centers. It was found that the addition of as few as 2 cells from a congenitally infected embryo could ultimately induce resistance to RSV in a culture of 10^6 sensitive cells (18). This finding supported the impression that a high proportion, and perhaps all, of the cells from congenitally infected embryos not only were infected but were continuously producing virus.

Having established a few parameters of congenital infection through study of parents and embryos, it was decided to extend the investigation, and study the fate of the embryos after hatching. The following questions were kept in mind during this portion of the study: (i) Did congenital infection establish immunological tolerance to the virus and did the tolerant chicks become the persistently viremic adults? (ii) Did the uninfected chicks become infected by contact? If so, when did they become infected and when were antibodies made? (iii) Which class of animal was more likely to develop leukosis, the congenitally infected or the contact infected?

To get significant numbers of lymphomatosis cases among the progeny, it was necessary to increase the population under study to a final total of 63 fertile females, 10 males, and about 800 progeny. The progeny were bled in staggered groups at various times after birth. An attempt was made to obtain at least two consecutive blood samples at an interval of several months from each of the progeny birds; as many as four samples each were obtained from about

100 of the birds. Each serum was tested for virus and antibody. In analyzing the results, the progeny were divided into two classes consisting of those coming from hens which were regular congenital transmitters (RIF(+) families) and those coming from hens which were not congenital transmitters (RIF(−) families).

The results on the persistence of viremia in congenitally infected birds can be described in a sentence: all those birds which were congenitally infected had a high level of viremia over the entire 7-month period in which progeny sera were tested. Of the noninfected chicks in contact with the congenitally infected birds, a few began to show a slight viremia during the early weeks of life, but involvement of a significant fraction of the population with viremia did not begin until 9 weeks after hatching.

The concentration of virus in the blood of all but a few of the contact infections remained several orders of magnitude lower than that of the congenitally infected birds. The number of birds with detectable viremia reached a maximum at 14 weeks and thereafter decreased. The probable reason for this decrease became evident when the antibody status of these birds was determined, and is discussed below.

None of the individuals known to be congenitally infected developed antibodies. In the RIF(+) families only those few siblings which had escaped congenital infection developed antibodies. The pattern of antibody development in the latter group was the same as that encountered in the progeny of hens which were *not* congenital transmitters (RIF(−) families).

In the RIF(−) families, passively transferred antibodies were found both in the yolk of the embryo and in the sera of 2-week-old birds in about $\frac{1}{50}$ the concentration present in their parents. Passively transferred antibody could no longer be detected when the chicks reached the age of 4 weeks, but actively produced antibody began to appear in some birds at 9 weeks of age. The proportion of birds with antibody then increased, with a particularly sharp increase occurring between 14 and 18 weeks. It will be recalled that it was at 14 weeks that a downturn occurred in the number of contact birds with viremia. It seems likely that this was related to the increase both in the proportion of birds with antibody and in the concentration of antibody in individual birds.

Perhaps the most striking aspect of these investigations was the clear indication that immunological tolerance to RIF was established by virtue of congenital transmission. In this respect the RIF system resembles congenital infection of mice with lymphocytic choriomeningitis virus (LCM). In the case of LCM it has been established that the congenitally infected mouse has a persistent viremia with no antibody production, thereby implying immunological tolerance (9, 22). As adults, tolerantly infected mice are unaffected by an intracerebral inoculation of LCM which kills previously uninfected mice. The survival of tolerant mice suggested that the disease produced by intracerebral inoculation of previously uninfected mice was the result of the immunological response of the host rather than the cytopathic interaction between virus and cell. It thus became a matter of great interest to determine whether lymphomatosis in chickens might also be the result of the immunological response of the organism to virus infection. If lymphomatosis had such an immunological basis, it seemed likely that the probability for developing the disease would be considerably higher in the contact birds than in the congenitally infected, immunologically tolerant birds.

In actual fact, the reverse of these expectations was found. The probability for developing visceral lymphomatosis proved to be six times higher in the congenitally infected birds than in the contact-infected birds. The level of the viremia in the congenitally infected birds did not diminish shortly before their death from visceral lymphomatosis, as might be expected if the disease were associated with an immunological response to infection. Therefore, the immunological hypothesis, at least in its simplest form, is not supported by the results. There remains the possibility that the disease is the result of the excessive proliferation of infected cells stimulated by antigens of various kinds other than those directly associated with RIF infection. If such were the case, the virus would have to be considered as a conditioning factor which merely increases the probability that a cell will become malignant under certain stimuli.

C. Experimental Establishment of Tolerance

The experiment of nature described above has been most fruitful in providing us with a picture of the natural history of a ubiquitous tumor

TABLE 3. *Role of early viremia in establishing tolerant infection to RIF*

Viremic history during first 3 weeks after hatching[a]		Fraction developing tolerant infection
1st week	3rd week	
3+	3+	10/10
2+	3+	6/7
−	3+	3/7
−	2+	0/6
−	−[b]	0/14

[a] 3+ = Heavy viremia (>10^5 infective units of RIF per ml plasma), 2+ = moderate viremia (10^2 to 10^5 infective units), and − = no viremia (<10^2 infective units).

[b] Viremia after 3 weeks.

virus, but it has also been useful in provoking a number of questions which can be answered only by direct experimentation. A question which has occupied much of our attention recently concerns the conditions which must be satisfied for the successful establishment of immunological tolerance. As a prelude to this work, it was necessary to carry out a growth curve of RIF in vitro (M. Feldman, *personal communication*). RIF is very slow to reach a constant level of virus production, requiring some 9 days. By way of contrast, RSV requires about 3 days (21, 23) and a cytocidal virus such as Newcastle disease virus (NDV) requires less than ½ day (19). Precise comparisons of the final rates of virus production cannot be made as yet since the experiments with RIF have not been carried out with the same precision as those with RSV and NDV. It is safe to say, however, that the ratio of free virus to cell-associated virus has been found to be even higher for RIF than for RSV (M. Feldman, *personal communication*), indicating that the release of the virus particle once it has attained infective maturity is very rapid indeed. Therefore, it is likely that RIF, like RSV, is completed at the cell surface.

To determine the conditions required to establish tolerant infection, embryos which had been incubated for 3 to 17 days were infected by various routes of inoculation. Infection was also carried out on chicks from 1 day to 6 weeks after hatching. The chicks were bled every other week from 1 to 17 weeks after hatching and the RIF and antibody contents of the sera determined. The results in Table 3 show that the basic requirement for the establishment of tolerance is that the virus must multiply to a high concentration in the infected chick within the first few weeks after hatching. Since this would require at least two cycles of growth in the animal and since the virus grows slowly, it would be necessary to infect the embryo at least a few days before hatching to reach a high level of virus multiplication during the first few weeks of life. Indeed, this is borne out by the results which show that there is great difficulty in establishing tolerant infection in chickens after hatching even when high concentrations of virus are inoculated into 1-day-old chicks.

IV. Discussion

The results of these experiments provide a clear picture of infection with RIF, an almost ubiquitous tumor virus. The prevalence of the virus would not be possible if it were as virulent for the host as are RSV or myeloblastosis virus, because inoculation of the more virulent agents into the embryo results in death of the chick within a few days or weeks after hatching. This lethality provides an explanation for the rarity of these viruses in the field. It also makes it perfectly clear that such "hothouse" strains of virus are unsuited for studying certain crucial aspects of the relationship between tumor virus and host. One of these aspects is of course the role of congenital transmission in producing immunological tolerance which has been adequately discussed above. Another aspect concerns the conditions which shift a well-nigh perfect symbiotic relationship between virus and host into a lethal one. It may well be that the RSV model for cellular alteration by changing the surface is also an appropriate one to describe the cellular changes in RIF infection *once the balance has been shifted.* In the case of RSV, however, multiplication of the virus is itself sufficient to cause a malignant change in cell behavior, but this is clearly not the case in RIF infection. There must be other factors which precipitate the malignant alteration and at present these can best be studied with the naturally occurring agent itself. Therefore, it seems reasonable to conclude that the studies of RSV infection at the level of the cell and RIF infection at the level of the organism will combine to provide a comprehensive picture of the viral carcinogenesis in the chicken, and perhaps in other animals as well.

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