the p-primary component of $\pi_{4n+2}(Sp(j))$ *is not zero for* $n \geqq j > l$. *The p-primary component of* $\pi_{4n+2}(SO(j'))$ *is not zero for* $4n + 2 \geqq j' \geqq 4l + 4$.

THEOREM 2. *Let p be an odd prime and let* $k \geqq 1$ *and* $i \geqq 3$. *Then the p-primary component of* $\pi_{2k(p-1)-1+i}(S^i)$ *is not zero. In fact,* $\alpha_k(i)$ *is of order p.*

Proof: Obviously $p\alpha_k(i) = 0$. Let $n = k(p - 1) + 1$ and let m be an integer such that $2m - 2n \geqq i - 3$. Then it follows from (B) and Lemma 3 that $i_*\zeta_*^{m-n}\alpha_k(2m - 2n + 3) = (-1)^{k+1}(m!/p)\,\partial\mathcal{E}_m \neq 0$. Thus $\alpha_k(i) \neq 0$. q.e.d.

We say that an element $\alpha \in \pi_q(S^s)$ is *decomposable* if α is the sum of some compositions $\beta_t \circ \gamma_t$ of $\beta_t \in \pi_r(S^s)$ and $\gamma_t \in \pi_q(S^r)$ for $q > r > s$. If α is decomposable, then so is $E\alpha$.

THEOREM 3. $\alpha_k(s)$, $s \geqq 3$, *is not decomposable.*

Proof: Consider (B) of $n = k(p - 1) + 1$ and $2m - 2n > s - 3$. Assume that $\alpha_k(s)$ is decomposable: $\alpha_k(s) = \Sigma\beta_i \circ \gamma_t$. Then $0 \neq (-1)^{k+1}(m!/p)\partial i_{0*}\mathcal{E}_m = i_{0*}i_*\zeta_*^{m-n}\alpha_k(2m - 2n + 3) = \Sigma(i_{0*}i_*\zeta_*^{m-n}E^t\beta_t) \circ E^t\gamma_t$, $t = 2m - 2n - (s - 3) > 0$. Since $E^t\beta_t$ belongs to a finite group $E^t\pi_r(S^s)$, $2n - 3 + s > r > s$ and since $\pi_{r+t}(SU(m)) \approx Z$ or 0 for $2m > r + t$, then $i_{0*}i_*\zeta_*^{m-n}E^t\beta_t = 0$ and thus $(m!/p)\partial i_{0*}\mathcal{E}_m = 0$. But this is a contradiction. Thus $\alpha_k(s)$ is not decomposable. q.e.d.

* This research was supported in part by the United States Air Force through the Office of Scientific Research of the Air Research and Development Command.

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*A VIRUS IN CHICK EMBRYOS WHICH INDUCES RESISTANCE IN VITRO TO INFECTION WITH ROUS SARCOMA VIRUS**

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Communicated by Robley C. Williams, June 13, 1960

When chick embryo cells are infected *in vitro* with Rous sarcoma virus (RSV) under the appropriate conditions they grow into foci of Rous sarcoma cells. The enumeration of these foci serves as an accurate assay for the infectious titer of a RSV preparation. In occasional sets of chick embryo cultures, however, the efficiency of infection with a standard RSV stock is reduced by a factor 50 or more. Once established, the resistance of such cultures to RSV infection is maintained indefinitely under all physiological conditions tested.

This state of cellular resistance, in which multiplication of RSV is prevented, is distinct from those physiological effects which merely suppress characteristic morphologic alterations in cells supporting multiplication of RSV.[1] The present paper describes the detection of resistant cultures and the characterization of the

agent responsible for inducing resistance. A quantitative description of the resistance phenomenon itself will be described in detail in another paper.

Materials and Methods.—Source of chick embryos: Embryos were obtained from the Kimber Farms Hatchery in Niles, California. Two hybrid strains of white leghorn chickens were used as the source for all the work described here, although additional confirmatory work has been carried out in two other strains. The 813 strain was used in routine assay work. Strain 13, a cross with a high incidence of lymphomatosis, was found to have a relatively high frequency of RSV-resistant embryos, and was the chief source strain in the investigations of resistance.

Primary chick embryo cultures: Fresh fertile eggs were delivered weekly and set biweekly. The incubated embryos were used to prepare cultures 10 days later. About 10 embryos on the average were pooled, the cells were suspended by trypsin treatment, and primary cultures prepared according to a procedure described in detail previously.[2]

For certain experiments it was necessary to prepare cultures from individual embryos. The procedure was similar to that employed for pooled embryos, except that smaller volumes of fluid were used. When pooled cultures were to be made from the cells of embryos which had been separately prepared for culture, each embryo contributed an equal number of cells to the total.

Secondary cultures and virus assay: The procedure for preparing secondary and tertiary cultures for use in the RSV assay was similar to that described before[2] with the following exceptions. In most of the work to be described, 10^6 cells were seeded in 5 ml of complete medium on a 50 mm petri dish. Infection of the cultures was made by adding 0.1 ml of virus stock to the cell suspension, and leaving it overnight while the cells settled and spread on the glass. The liquid medium was then removed and agar medium added. Rous sarcoma foci were counted 6 days later. Complete medium for secondary cultures was composed of the following ingredients in the indicated proportions. Mixture 199 salt and nutrient solution—81; tryptose phosphate broth—10; calf serum—5; chicken serum—2; 2.8 per cent NaH CO_3—2. This was modified in primary cultures by doubling the concentration of calf serum and making a compensatory decrease in the volume of 199.

The modified procedure had several advantages. The seeding of 10^6 cells rather than the 3 to 5 $\times$ 10^5 previously used resulted in cultures which were able to grow quickly into a confluent sheet more consistently. The addition of virus to cells which had not yet attached to the glass made it possible to obtain large foci with the higher cell concentration. If the addition of virus was delayed until the day following the seeding of 10^6 cells, the foci were smaller, and sometimes barely visible. The overnight incubation of virus with suspended cells permitted more complete adsorption than the 40 min adsorption period used in the earlier work. Since there is no significant virus production in the first day after infection, there was no danger of encountering secondary foci from virus released from infected cells during overnight incubation in fluid medium. The revised procedure was also found to be of greater convenience than the original procedure since secondary cultures did not have to be prepared until immediately before use; their preparation and use, therefore, became in effect, a single operation.

Standard RSV stock: A stock was prepared from Bryan's high-titered RSV.[2] It was kept frozen at −60°C in tubes containing 1.0 ml and was assayed daily to provide a routine measure of sensitivity to RSV infection of cultures used in all experiments. The stock usually titered between 4 and 10 × 10^6 focus forming units (FFU) per ml in the revised assay procedure.

Antiserum to RSV: Antiserum was obtained from chickens which had recovered from tumors induced with 10 to 100 FFU of RSV stock. After regression of tumors, the chickens were hyperimmunized by inoculation with 10,000 FFU of RSV stock, and bled 3 weeks later.

Removal of agar medium. In some experiments it was necessary to assay the materials released by chick embryo cells. Since the cell sheets tended to peel off the glass in a few days, it frequently was necessary to maintain cultures under agar. To remove the agar, 3.0 ml of 199 was added for 2 to 3 hr. The softened agar was then ejected into a beaker by a quick, jarring movement of the petri dish. The agar and fluid were mixed by pipetting, and centrifuged at 1,500 RPM for 2 to 3 min. The supernatant fluid was used as the source of the material released by the cells.

Results.—The occurrence of resistant cultures: Despite the fact that the eggs used for chick embryo cell cultures were obtained from a single source, there was a discontinuous variation in the sensitivity of the biweekly groups to infection with a standard RSV stock. The virus assays fell into two classes. Most of the assays of the stock virus preparation gave titers between 4 and 10 × 10^6 FFU per ml of stock virus. From time to time, however, the virus titer dropped to 10^5 FFU per ml or less. Once this resistance to RSV infection was encountered in a group of secondary cultures, all subsequent transfers from that group remained resistant to RSV infection. Cultures which were highly resistant to RSV infection were fully sensitive to infection with Newcastle disease virus.

An investigation was undertaken of the nature of the resistance to RSV infection. Initial studies were concerned with the effect of physiological changes on the sensitivity of chick cells to RSV. Variations in pH, temperature, cell concentration, constituents of the medium and exposure to light failed to induce stable resistance to RSV in chick embryo cultures, although transient effects on the morphological expression of foci were observed, similar to those described previously.[1]

It was noted that chick embryo pools which proved to be resistant to RSV as secondary cultures, were fully sensitive if challenged with RSV immediately after the cells had been explanted from the embryo. It was therefore evident that the resistance of the pooled secondary cultures had its onset during the 3 to 4 days of incubation which intervened between the preparation of the primary and secondary cultures. This suggested that the resistance might be conferred on the pooled culture as a whole by the cells from one or more of the constituent embryos. To determine whether this was so, separate primary cultures were prepared from individual embryos. Aliquots of cells from the individual embryos were also pooled in various combinations with cells from other individuals. Some of the cultures were immediately infected with RSV, and the remainder transferred to make secondary cultures at 3 or 4 days. Some of the secondary cultures were infected with RSV and the others transferred again at 3 or 4 days. By repeating

this procedure the sensitivity of the individual embryo cultures could be determined for an unlimited number of successive transfers. It was also possible to determine whether there was a correlation between the occurrence of resistance in an individual embryo culture and its occurrence in pooled cultures containing cells from that embryo.

The results of three such experiments are summarized in Tables 1, 2, and 3 which illustrate various features of the resistance phenomenon. Each table is divided into a section for individual embryo cultures and a section for pooled embryo cultures. Those individual embryos which remained sensitive to RSV infection during the course of an equal number of successive transfers are grouped together. Cultures which fell in the usual range of high sensitivity to infection with RSV are assigned a relative sensitivity value of 1. An average was calculated of the RSV titers from all the sensitive cultures of a given passage, and this was used as the baseline for assigning relative sensitivity values to the resistant cultures of that passage. Those cultures which gave an RSV titration one-tenth or less than that of the sensitive cultures were considered resistant, and their relative sensitivity values are italicized.

TABLE 1

THE RELATIVE SENSITIVITY TO RS. INFECTION OF CULTURES DERIVED FROM INDIVIDUAL EMBRYOS AND CORRESPONDING MIXED POOLS

Individual embryo, no.	Total no. embryos in class	Passage number 1	2	3	4	5	6	7
2	1	*<0.01**(+)	*0.01*(+)	*<0.01*	*<0.01*	*<0.02*	*<0.02*	*<0.01*
10	1	*<0.001*(+)	*<0.001*(+)	...	...	...	...	...
16	1	1(−)	1(−)	1	1	1	1	1
19	1	1(−)	1(−)	1	1	1	1	*0.02*
20	1	1(−)	1(−)	1	1	1	*0.01*	...
1, 3, 4, 5, 9, 11, 12, 13, 14, 15, 17, 17, 18, 21	13	1(−)	1(−)	...	...	...	...	...
Pool containing embryos, no.								
2–5	4	1(+)	*<0.001*(+)	*<0.01*	...	...	...	...
9–12	4	1(+)	*<0.001*(+)	*<0.01*	...	...	...	...
13–16	4	1(−)	1(−)	1	...	...	...	...
17–21	5	1(−)	1(−)	1	...	...	...	...
1–10	10	1(+)	*<0.001*(+)	*<0.01*	...	...	...	...
11–21	11	1(−)	1(−)	1	...	...	...	...
1–21	21	1(+)	*<0.001*(+)	*<0.01*	...	...	...	...

* = Relative sensitivity to RSV infection.

A. Six primary cultures were made from each individual embryo and constituted passage number 1. The remaining cells from each embryo were mixed with equal numbers of cells from other embryos in various combinations to make pooled cultures. Two cultures were challenged immediately with a 1:20 dilution of RSV stock and 2 with 1:1000 dilution. The remaining 2 cultures were subdivided into 6 cultures at 4 days; 4 of the cultures were in turn challenged with RSV and 2 kept for further transfer. This operation was repeated every 3 to 4 days to determine the sensitivity to RSV infection. The italicized values indicate resistant cultures.

B. The medium was harvested from the unchallenged cultures of passages 1 and 2 and frozen. At a later time, it was tested for the presence of a resistance inducing factor (RIF) by adding 0.1 ml to the medium of sensitive cultures, transferring the cultures twice at intervals of 3 to 4 days and challenging with RSV after the second transfer. Cultures containing RIF are designated (+); those containing no RIF are designated (−).

Some cardinal features of the resistance phenomenon are illustrated in Table 1. In the primary cultures, 2 of the 21 individual embryos were found to be resistant to infection with RSV immediately after explantation. The pooled cultures which contained cells from these individual embryos were sensitive to RSV infection

immediately after the pool was composed, but became resistant when challenged at the first transfer. Once a culture had become resistant it remained resistant through all subsequent transfers. The degree of resistance varied somewhat but in each case reached a level at least 50 times that of the average sensitive culture.

Of the individual embryo cultures listed in Table 1 which were initially sensitive to RSV infection, two became resistant in the 6th or 7th passage. A similar occurrence is illustrated in Table 2, where cultures from embryo no. 5 and from the pool containing embryos no. 13–16 became resistant in the 6th passage. The pool which contained embryo no. 5 did not become resistant during the course of the experiment.

TABLE 2

THE RELATIVE SENSITIVITY TO RSV INFECTION OF CULTURES DERIVED FROM INDIVIDUAL EMBRYOS AND CORRESPONDING MIXED POOLS

Individual embryos, no.	Total no. embryos in class	Passage number 1	2	3	4	5	6
5	1	1*	1	1	1	1	*0.01*
2, 9, 11, 14, 17	5	1	1	1	..	..	..
8, 20	2	1	1	1	1	..	..
1, 3, 4, 6, 7, 10, 12, 13, 15, 16, 18, 19, 21, 22	14	1	1	1	1	1	1
Pool containing embryos, no.							
1–4	4	1	1	1	..	..	..
5–8	4	1	1	1	1	1	1
9–12	4	1	1	1	1	1	1
13–16	4	1	1	1	1	1	*<0.001*
17–22	6	1	1	1	..	..	..
1–12	12	1	1	1	1	1	..
13–22	10	1	1	1	..	..	..
1–22	22	1	1	1	..	..	..

* Relative sensitivity to RSV infection.
See legend (*A*) in Table 1 for procedure.

Some individual embryo cultures which were initially sensitive to RSV infection became resistant after only one or two transfers and the corresponding pool became resistant at the same time. This is illustrated in Table 3 where individual and pooled cultures containing cells from embryo no. 2 became resistant in the 3rd passage. All the situations described above have been encountered re-

TABLE 3

THE RELATIVE SENSITIVITY TO RSV INFECTION OF CULTURES DERIVED FROM INDIVIDUAL EMBRYOS AND CORRESPONDING MIXED POOLS

Individual embryo, no.	Total no. embryos in class	Passage number 1	2	3
7	1	*0.02**	*0.01*	*0.01*
20	1	1	1†	*0.01*
1–6, 8–19	18	1	1	1
Pool containing embryos, no.				
1–4	4	1	1	1
5–8	4	1	*0.01*	*<0.01*
9–12	4	1	1	1
13–16	4	1	1	1
17–20	4	1	1†	*0.1*

* Relative sensitivity to RSV infection.
† delays in appearance of foci and in the acidification of medium in heavily infected cultures.
See legend (*A*) in Table 1 for procedure.

peatedly. Thus, the resistance phenomenon can be divided into three categories with respect to time of onset of resistance in individual and pooled embryo cultures. There are individual embryos which (*a*) are resistant immediately after explanation, (*b*) become resistant at the 2nd or 3rd passage, (*c*) become resistant only after 6 or 7 passages. The respective pools containing these individuals (*a*) become resistant only in the 2nd passage, (*b*) become resistant at the same time as their constituent individuals, (*c*) do not become resistant at the same time as their constituent individuals. Whether the pools of category (*c*) would have become resistant if cultivation had continued is unknown.

Demonstration of a Resistance-Inducing Factor (RIF) in the Medium of Resistant Cultures.—The observation that cells of a single resistant embryo could confer resistance on a pool containing a large majority of cells from sensitive embryos could not be explained by selective growth of the resistant cells since all cultures regardless of source were found to grow at the same rate. The possibility that the resistance of the pool could result from a physiological change in the medium conditioned by the presence of cells from a resistant individual was ruled out by experiments which showed that even the isolated clones derived from such pools retained their resistance. Therefore, it seemed likely that the resistance spread from the resistant cells to the sensitive cells.

The most direct means of spread was through the medium, and an attempt was made to detect the responsible agent. In a typical experiment, medium was removed from both resistant and sensitive individual embryo cultures. The medium was centrifuged to remove cell debris and 0.1 ml added to sensitive cultures. The cultures were transferred twice at intervals of 3 or 4 days, and challenged with RSV after the second transfer. The results of a typical experiment are presented in Table 4 where it is shown that the medium of resistant cultures did indeed contain a factor which induced resistance to RSV in cultures previously sensitive to RSV. This factor will be referred to as RIF, which is the abbreviation of resistance inducing factor. A stock of RIF was made by harvesting the medium from a highly resistant culture at daily intervals. The stock was stored at a temperature of −60°C.

TABLE 4

THE PRESENCE OF A RESISTANCE-INDUCING FACTOR (RIF) IN THE MEDIUM OF RESISTANCE CULTURES

Medium obtained from	Dilution of challenge RSV	No. of foci	Relative sensitivity
Resistant culture	1:25	99,84	*0.0036*
Sensitive culture	1:1000	473,562	1.0

Medium was removed from RSV-resistant and RSV-sensitive primary cultures and 0.2 ml of each medium was added to the medium of 2 freshly-seeded sensitive secondary cultures. The secondary cultures were transferred at 4 days and transferred again 3 days later to make 4 cultures. Two cultures were challenged with each of the indicated dilutions of RSV.

To determine whether RIF was invariably associated with the medium of resistant cultures, the medium obtained from the first and second passage of each culture of the experiment in Table 1 was tested for its ability to induce resistance in sensitive cultures. The sensitive test cultures were challenged with RSV at the second transfer after their presumptive exposure to RIF, and those which had a 20-fold or greater reduction in RSV titer were considered resistant. The results are presented in Table 1. Cultures whose media induced resistance (i.e., con-

tained RIF) are designated (+) and those whose media failed to induce resistance are designated (−). It is apparent that the medium of all resistant cultures contained RIF. The pooled primary cultures, which were themselves sensitive to RSV, but which were to become resistant in the succeeding transfer also released RIF into the medium. Unquestionably the presence of RIF in the medium of the pooled cultures was due to the presence of cells from a resistant individual, and the spreading infection accounted for the resistance displayed in the subsequent passage. None of the cultures which remained sensitive to RSV through the early passages released detectable amounts of RIF. The results, therefore, show a perfect correlation between the presence of RIF and the onset of resistance to RSV infection.

The assay of RIF: In order to carry out quantitative work with RIF it was necessary to establish an assay procedure. Serial dilutions of media containing RIF were made, and each dilution added to several sensitive cultures. The cultures were transferred and challenged with RSV at various times after addition of RIF. The results of this experiment are presented in Table 5. It can be seen that

TABLE 5

SPEED OF ONSET OF RESISTANCE TO RSV IN CULTURES INFECTED WITH RIF (DAYS AFTER RIF INFECTION)

RIF dilution factor	1st passage				2nd passage		3rd passage
	0 days	2 days	5 days	8 days	5 + 3 days	5 + 6 days	5 + 6 + 4 days
10^0	1*	0.5	*0.05*	*0.02*	*0.004*	*0.008*	*<0.001*
10^1	1	0.5	*0.1*	*0.03*	*0.008*	*0.008*	*<0.001*
10^2	1	1	1	*0.07*	*0.004*	*0.001*	*<0.001*
10^3	1	1	1	1	*0.03*	*0.004*	*<0.001*
10^4	1	1	1	1	1	*0.04*	*<0.001*
10^5	1	1	1	1	1	1	*<0.001*
Control (no RIF)	1	1	1	1	1	1	1

* Relative sensitivity to RSV infection.

Separate groups of 20 secondary chick embryo cultures were infected with 0.1 ml of each of 5 10-fold dilutions of RIF. One group was exposed only to the regular medium. Four cultures in each group were challenged with RSV on the indicated days. On the 5th day, 4 of the unchallenged cultures in each group were transferred to make 10 cultures, of which 4 were exposed to RSV challenge on the 8th (5 + 3) and 11th (5 + 6) days of the experiment. The 2 remaining cultures were transferred again on the 11th day and challenged with RSV 4 days later (5 + 6 + 4).

a mild resistance first became demonstrable at 2 days in those cultures exposed to undiluted RIF and to a 1:10 dilution of RIF. The resistance became stronger in the next few days and was strikingly apparent at dilutions as high as 10^{-3} after a second transfer of the treated cultures. A third transfer elicited resistance at the highest dilution tested.

Figure 1 illustrates a more detailed assay of the resistance to RSV induced by a RIF preparation as a function of the number of transfers after exposure to RIF. Twofold dilutions of RIF were used to infect sensitive cultures, and the cultures were transferred at intervals of 3 to 6 days. At each transfer, an aliquot of cells at each RIF dilution was set aside for challenge with RSV. It can be seen that there is about a 100-fold increase between the first and second passage in the dilution of RIF which induces resistance; about a 30-fold increase between the second and third passage; and a further 20-fold increase between the third and fourth passage. Further transfer failed to reveal the onset of resistance at higher dilutions. Therefore, a stable endpoint was reached at a dilution of 1:130,000. Since

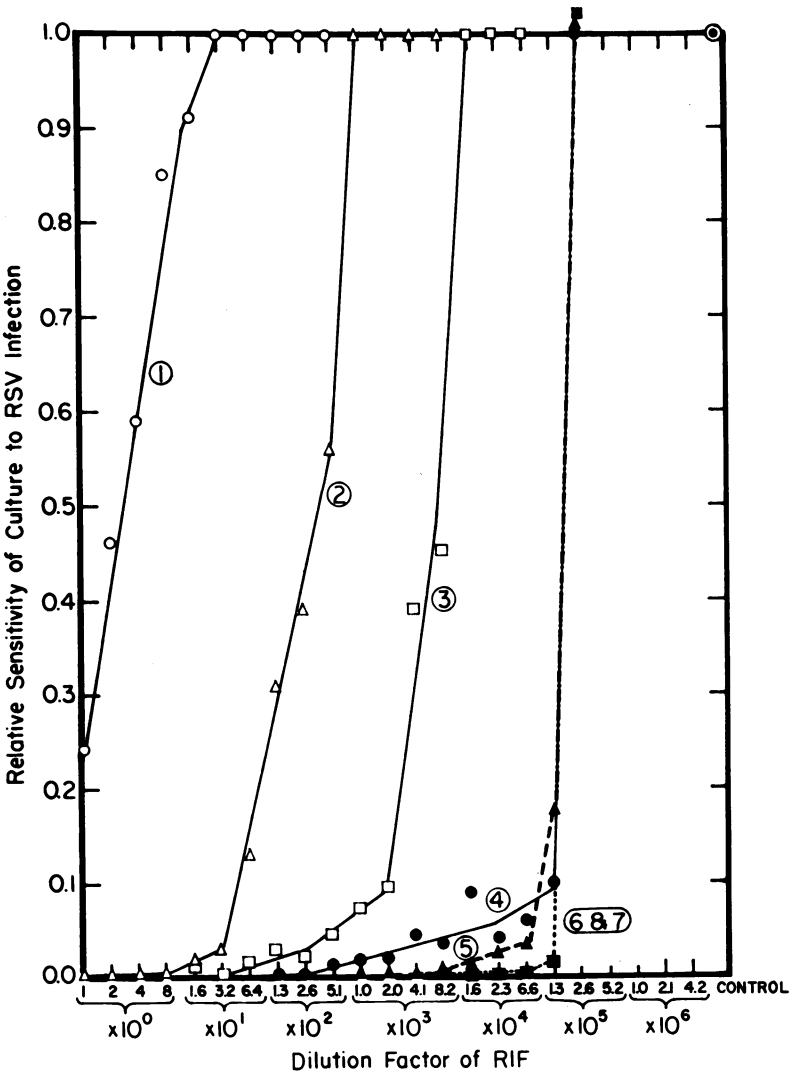

FIG. 1.—A series of twofold dilutions of RIF stock were made in M199 and 0.1 ml. of each dilution added to 2 chick embryo cultures. At 3- to 4-day intervals these were subdivided into 6 cultures, 4 of which were challenged with RSV and 2 of which were used for further transfer. The passage number at which the cultures were challenged with RSV are indicated on the graph. At the 6th passage, the media of the 9 highest dilutions of RIF, and of the control, were tested for the presence of RIF (see text for results of RIF test of medium).

0.1 ml was the volume of the initial inoculum, the titer of RIF in the stock preparation was approximately 1.3×10^6 infectious units per ml.

At the 6th passage, the agar medium was removed from cultures which had been infected with the 9 highest RIF dilutions, as well as from the control cultures. A fluid extract of the agar medium was added to the medium of fresh sensitive cultures. The cultures were transferred twice and challenged with RSV to determine whether RIF had been present in the extract of the agar medium. It was found

that all the resistant cultures (those which had been infected with dilution factors of up to 1.3×10^5 of the RIF stock) released RIF into the medium, as indicated by the fact that medium obtained from these cultures induced resistance in sensitive cultures after the second transfer. None of the sensitive cultures released RIF. This again demonstrated the association between resistant cultures and the presence of RIF.

The results of this experiment also provided unequivocal evidence for the multiplication of RIF. This conclusion is best exemplified by considering the cultures initially infected with the 1.3×10^5 dilution factor of RIF stock. Resistance was not manifest in these cultures until the 4th passage. The medium removed from the 6th passage, however, was able to induce resistance within 2 passages. The highest dilution factor of the original stock able to induce a high level of resistance by the 2nd passage was 3.2×10^1. Therefore, there had been an increase in potency over the initial inoculum of at least 4,000-fold. In addition to this factor it is necessary to include in the calculated degree of multiplication of RIF, the subdivision of each culture into three at each transfer, resulting in a dilution factor of the cells of about 700 in the 6 transfers, and the initial dilution factor of 50 when 0.1 ml of inoculum was added to 5.0 ml of medium. Therefore, the agent must have multiplied by a factor of over 10^8 in 6 passages.

The nature of RIF: The ability of RIF to infect, multiply in, and be released from chick embryo cells suggested that it was a virus. Among the common viruses of chickens, perhaps the most ubiquitous is the agent of lymphomatosis. Suspicion of a relationship to RIF focused on lymphomatosis not only because of the wide distribution of the latter in nature, but also because it is known to be transmitted *in ovo* without harmful effect to the embryo.[3] Since lymphomatosis virus is immunologically related to RSV[4] an experiment was carried out to determine whether RIF could be neutralized by antiserum to RSV. In an accompanying experiment, the effectiveness of the antiserum against its homologous antigen, RSV, was evaluated.

TABLE 6

NEUTRALIZATION OF RIF AND RSV BY ANTISERUM TO RSV

A			B	
Test for Anti-RIF Activity			Test for Anti-RSV Activity	
RIF dilution factor	Serum dilution	RSV titer after challenge $\times 10^{-6}$	Serum dilution	RSV titer $\times 10^{-6}$
10^0	1:50	2.0	1:200	0.012
	1:200	*0.62*	1:400	0.035
	1:800	*0.04*	1:800	0.043
	1:1600	*0.04*	1:1600	0.068
	no serum	*0.03*	1:3200	0.33
10^1	1:50	4.0	no serum	3.25
	1:200	2.8		
	1:800	2.5		
	1:1600	*0.3*		
	no serum	*0.4*		
No RIF	1:50	3.7		
No RIF	no serum	3.0		

Mixtures were made of RIF and antiserum to RSV at the appropriate dilutions. The mixtures were incubated 40 min, at 37° and 0.1 ml added to chick embryo cultures. These were transferred twice at intervals of 3 and 5 days and challenged with RSV after the second transfer. RSV titers less than 0.7×10^6 indicate the presence of large amounts of unneutralized RIF in the original preparation. Titers of 2.5×10^6 or higher are taken to indicate full RIF activity.

Nine-tenths ml of each serum dilution was added to 0.1 ml of undiluted RSV stock. The mixtures were incubated 40 min at 37°, appropriate dilutions were made, and 0.1 ml added to chick embryo cultures. The number of Rous sarcoma foci was recorded at 7 days.

The experimental procedures and the results of this experiment are presented in Table 6. Dilutions of antiserum as high as 1:1600 reduced the infectivity of RSV by a factor of 50 or greater and even the 1:3200 dilution reduced the RSV titer by a factor of 10. Dilutions of the antiserum up to 1:800 neutralized the 1:10 dilution of RIF to some extent as indicated by the loss of its capacity to interfere with infection by RSV. The 1:800 serum dilution had no measurable effect on the activity of the undiluted RIF. A 1:200 dilution of the antiserum reduced the interfering capacity of an undiluted suspension of RIF to the level of a 1:10 dilution of the same RIF stock, showing that even at this relatively high serum concentration approximately 10 per cent of the RIF population escaped neutralization. These results indicate an immunological relationship exists between RIF and RSV, but also suggest that the agents can be distinguished immunologically. The immunological relationship between RIF and RSV has been recently confirmed by the demonstration that antisera obtained from chickens immunized with RIF neutralized RSV.

An experiment was carried out to determine whether RIF could be sedimented in gravitational fields adequate to sediment RSV. A stock of RIF was clarified by low speed centrifugation and then centrifuged in a field which varied from a minimum of about 50,000 × *g* at the top of the centrifuge tube to about 110,000 × *g* at the bottom. Both the resuspended pellet and supernatant fluid, as well as the original stock were tested for resistance inducing activity.

The results in Table 7 show that less than one per cent of the initial activity remained in the supernatant fluid, while the pellet showed a high degree of activity.

TABLE 7

ULTRACENTRIFUGATION OF RIF

Dilution factor of sample	RSV Titer × 10^{-6}		
	Original	Pellet	Supernate
10^0	*0.03**	*0.03*	*0.08*
10^1	*0.02*	*0.08*	**1.5**
10^2	*0.02*	*0.10*	5.6
10^3	*0.78*	**2.2**	6.3
10^4	**2.6**	4.8	7.0

*Titer of RSV on culture determined at the 2nd passage after introduction of RIF.

Titer of RSV on control preparation unexposed to RIF = 4.7 × 10^6:
Italics = Highly resistant culture
Boldface = Partly resistant culture

Thirty ml of RIF stock was centrifuged in the #30 rotor of the Model E preparative Spinco ultracentrifuge at 30,000 RPM for 30 min. All but about 2 ml of supernatant fluid was drawn off and 18.5 ml of 10% calf serum in 199 added to resuspend the pellet. The original stock, supernatant fluid, and resuspended pellet were assayed for RIF activity. A control culture containing no RIF was included in the experiment.

Because of the statistical limitations of any end point assay employing tenfold dilutions, it is not possible to state with precision the amount of RIF recovered in the pellet, but it is evident that there is almost 100 times more activity than in the supernatant fluid. The pellet proved to be somewhat less infectious than the original material, indicating that RIF was partially inactivated by centrifugation. We have found that a similar loss of infectivity accompanies the centrifugation of RSV.

Preliminary experiments[5] show that RIF also resembles RSV—and presumably lymphomatosis virus as well—in its thermal and ether sensitivities, and in its high resistance to inactivation by ultraviolet light. It is distinct from the adenovirus-

like agent isolated from chick embryo cultures[6] in its failure to damage cultures of liver parenchyma cells.

The assay of lymphomatosis virus by induction of resistance to RSV: In establishing a relationship between RIF and lymphomatosis virus it would, of course, be important to determine the manifestations of RIF infection in chickens. The test of the ability of RIF to cause the lymphomatosis disease complex in chickens can only be carried out in special strains of chickens and requires 9 months to complete.[7] Pending the outcome of such a trial, it was decided to carry out the reverse experiment and test several strains of lymphomatosis virus for their ability to induce resistance to RSV in cultures of chick embryo cells.

Nine ampoules of cell-free tissue extracts derived from chickens with lymphomatosis were obtained from Dr. B. R. Burmester of the Poultry Regional Laboratory, East Lansing, Michigan. Three of the extracts were from chickens involved in outbreaks of lymphomatosis in widely separated localities, and had been shown to reproduce the disease when inoculated into sensitive chickens. The remaining six ampoules were from various serial passages of a tumor derived from a single outbreak (RPL12) of lymphomatosis. Two of the cell-free extracts had failed to produce a significant incidence of lymphomatosis when inoculated into chickens, and were included in the *in vitro* assay to serve as controls.

An aliquot of each ampoule was added to two cultures of chick embryo cells which were sensitive to RSV infection. In addition, two cultures were inoculated with our own stock of RIF obtained from a naturally occurring chick embryo infection, and another two cultures remained uninoculated. The sensitivity of cultures to RSV infection was assayed at each of four subsequent cell transfers. There were no prominent morphological alterations in the cultures infected with the lymphomatosis viruses, although there was a tendency for the cells to grow in disorganized patterns and to become granular and refractile. These changes also characterized RIF-infected cultures after several transfers.

The quantitative results of this experiment presented in Table 8, show that almost every extract which had proved capable of inducing lymphomatosis in chickens induced a significant measure of protection against RSV after only a

TABLE 8

ASSAY OF LYMPHOMATOSIS VIRUS FOR ABILITY TO INDUCE RESISTANCE TO RSV *in vitro*

Sample tested for RIF activity		Year prepared	Lymphomatosis ID 50 in chickens	Passage number			
				1	2	3	4
RPL	12	1953	10^{-7}	0.5*	*0.03*	*0.02*	*0.02*
RPL	12	1957	10^{-4}	*0.08*	*0.02*	*0.02*	*0.007*
RPL	12	1958	10^{-4}	0.5	*0.02*	*0.003*	*0.002*
RPL	12	1958	10^{-3}	*0.05*	*0.006*	*0.002*	*0.003*
RPL	12	1959	negative	0.7	1	0.8	*0.02*
RPL	12	1960	negative	0.9	1	0.6	*0.005*
RPL	16	1953	10^{-1}	**0.2**	*<0.01*	*0.002*	*0.003*
Akron	1	1959	undiluted	**0.2**	*0.003*	*<0.001*	*0.002*
Hickory	5	1959	"infectious"	*0.1*	*0.004*	*<0.001*	*<0.001*
RIF stock		1960	unknown	1.0	*0.02*	*0.03*	**0.17**
Black control		—	—	1.0	1.0	1.0	1.0

* Relative sensitivity to RSV infection:
Italics = Highly resistant culture
Boldface = Partly resistant culture

One-tenth ml of each of the indicated lymphomatosis virus samples were added to 2 chick embryo cultures. At 3 to 4 day intervals these were subdivided into 6 at 3 days, of which 4 were challenged with RSV and 2 used for further transfer. Controls consisted of cultures infected with the regular laboratory RIF stock and uninfected cultures.

single cell transfer; by the second transfer, cultures treated with every one of the lymphomatosis-inducing extracts were strongly resistant to RSV infection, and the cultures retained this resistance in subsequent transfers. Most of the lymphomatosis positive virus samples were, in fact, more potent than the standard RIF stock when measured by the speed of onset of the resistance induced in chick embryo cultures.

The two tissue extracts which had produced no disease in chickens were without effect on the RSV sensitivity of the chick embryo cultures for 3 transfers. At the 4th cell transfer, however, the cultures became resistant to RSV infection. The delayed onset of resistance suggested, on the basis of the standard curves of Figure 1, that these two samples contained less than 1/10,000 as much virus as some of the samples which produced lymphomatosis in chickens. Thus, the failure of these samples to produce a significant increase over the background incidence of lymphomatosis after injection into chickens might have been due to their low virus content.

These results lend support to the suggestion that RIF is related to lymphomatosis virus. Aside from the implications of these results for the identification of RIF, they indicate that lymphomatosis can be easily detected by tissue culture methods. Not only do these methods appear to surpass the animal inoculation techniques in speed and efficiency, but they are apparently more sensitive for the detection of low concentrations of virus.

The presence of RIF in ovo: It was shown above that the first striking evidence for the induction of resistance to RSV infection in sensitive chick embryo cultures appears between 2 and 5 days after their deliberate infection with RIF. Therefore, the fact that the cells of some individual embryos were highly resistant to RSV infection at the time of their explantation suggested most of the cells had already been infected for several days prior to explantation.

Furthermore, detailed studies on the nature of the resistance to be reported in another paper indicate that almost every cell obtained from a resistant embryo, and capable of growing in tissue culture, is infected with RIF. In view of these observations, an attempt was made to demonstrate the presence of fully infectious RIF in various tissues and in the allantoic fluid of resistant embryos immediately after their removal from the egg.

Eighteen 10-day old embryos of strain 13 were used. The allantoic fluid was removed from each and frozen. Before dispersing the embryo cells with trypsin for the preparation of primary cultures, one leg and the pooled abdominal organs of each embryo were removed and frozen. The primary, secondary, and tertiary cultures of each embryo were challenged with RSV. It was found (Table 9a) that the primary cultures of 2 of the 18 embryos (no. 10 and no. 14) were highly resistant to RSV infection. One of the embryos (no. 9) was slightly resistant to RSV in primary culture but became highly resistant in secondary culture. Cultures derived from the other embryos remained sensitive to RSV infection through all three transfers.

The legs and organs of the 3 resistant embryos and of one sensitive embryo were triturated in a cold mortar and pestle. The tissue suspensions were centrifuged, and the supernatant fluids frozen and thawed to destroy any intact cells which might remain. Aliquots were added to RSV-sensitive chick embryo cultures, and the sensitivity of these cultures to RSV infection was determined after one and two

TABLE 9

THE PRESENCE OF RIF IN OVO

A: Test of Individual Embryo Cultures for RSV Resistance					B: Test of Homogenates from Individual Embryos for RIF Activity				
Individual embryo, no.	Total no. embryos in class	Passage number 1	2	3	Embryo	Source of material	Passage number 1	2	3
9	1	**0.25***	*<0.01*	*<0.01*	# 9	Leg	1	*0.05*	...
						Organs	1	*0.03*	...
						All fl.	1	*0.08*	...
10	1	*0.02*	*0.02*	*0.02*	# 10	Leg	*0.1*	*0.01*	...
						Organs	*0.07*	*0.01*	...
						All. fl.	1	*0.03*	...
14	1	*0.1*	*0.02*	*0.02*	# 14	Leg	**0.2**	*0.02*	...
						Organs	**0.3**	*0.03*	...
						All fl.	1	**0.3**	*0.03*
1–9, 11–13 15–18	15	1	1	1	# 15	Leg	1	1	...
						Organs	1	1	...
						All. fl.	1	1	1

* Relative sensitivity to RSV infection.

A. Eighteen individual embryos were removed from the egg. The allantoic fluid, a single leg, and the abdominal organs were frozen. The remainder of the carcass of each was used to prepare cultures which were tested for RSV-resistance in 3 successive passages.

B. The frozen tissues from the 3 embryos which had yielded resistant cultures and from one embryo which had yielded sensitive cultures were triturated with a mortar and pestle in 10 volumes of tris buffer containing 10 per cent calf serum. The homogenates were centrifuged at 1,500 RPM in an International clinical centrifuge for 5 min and the supernatant fluids withdrawn. These were quickly frozen and thawed to destroy intact cells, and tested for the presence of RIF by infecting sensitive cultures with 0.2 ml and challenging with RSV at the indicated passages. The allantoic fluids were tested without prior trituration or centrifugation.

and, in some cases, three transfers. An aliquot of the homogenate was added to complete medium in petri dishes containing no cells to determine whether there were any living cells present as evidenced by their ability to spread on glass; no living cells were found. The allantoic fluid samples were tested for the presence of RIF without prior centrifugation.

The results of this part of the experiment are in Table 9B. It may be seen that the leg and organ extracts obtained from the two embryos which gave highly resistant primary cultures induced resistance to RSV in sensitive cultures after a single transfer of the cultures. The allantoic fluids from these embryos, and all the materials obtained from embryo no. 9 produced resistance in cultures only after the cultures had undergone two transfers. Extracts from embryo no. 15, which had yielded RSV-sensitive cultures when its cells were cultivated, failed to induce resistance when added to the medium of other sensitive cultures.

These results show that the embryos which are the source of highly resistant primary cultures produce relatively high concentrations of RIF *in ovo*. The virus concentration in the allantoic fluid is much lower than in the embryo itself. An embryo which yields partially resistant primary cultures has relatively low but clearly demonstrable concentrations of RIF at the moment of explantation showing that the agent is actively multiplying *in ovo*.

Discussion.—The inhibition of RSV multiplication in chick embryo cells by prior infection with RIF may be regarded as a form of interference between animal viruses. In contrast to most cases of interference between animal viruses,[8] however, the inhibition by RIF appears to be specific for RSV. This conclusion is based not only on the observation mentioned above that chick embryo cultures resistant to RSV are fully sensitive to Newcastle disease virus, but on the fact that in several years of experience with infection of chick embryo cultures with a

variety of cytocidal viruses we have encountered no incidents of chick embryo cultures highly resistant to infection with these agents.

In all the criteria examined, RIF is indistinguishable from the agent of avian lymphomatosis. Both are widely distributed among apparently healthy hens and their embryos.[9] Neither produces any overt cytopathic effect in tissue culture, although both cause increased cellular granularity and disorganization of the whorled pattern which characterizes normal chick fibroblast growth. Like RIF, lymphomatosis virus has been reported to cross react immunologically with RSV,[4] and preliminary studies[5] indicate that RIF has the physical properties shared by all the known chicken tumor viruses.

Conclusive proof of the identity of RIF must await the outcome of infection of chickens. Regardless of the nature of RIF, however, the technique for its assay by inhibiting the growth of RSV has been shown to be applicable to the assay of lymphomatosis virus. The success in detecting lymphomatosis virus suggests that the principle of the interference technique might be useful in the assay of viruses associated with other malignancies. The viruses associated with mouse leukemia and mouse mammary carcinoma come most sharply to mind, since they resemble lymphomatosis virus in their failure to produce an overt cytopathic effect *in vitro*. It would appear necessary, however, to adapt at least one mouse tumor virus to play the role of a sentinel virus similar to the role played by RSV in the detection of lymphomatosis virus. If such a technique proved successful for the detection of known tumor viruses, an effort would be warranted to adapt it as a tool for investigating the cause of tumors of unknown etiology.

Summary.—Sporadic resistance to infection *in vitro* with Rous sarcoma virus (RSV) was encountered among pools of cells obtained from several chick embryos. It was found that the resistance encountered in the pools was always associated with the presence of one or more embryos whose cells were resistant when cultivated separately. An agent could be invariably isolated from the medium of resistant cultures, which induced resistance to RSV but not to Newcastle disease virus when added to cultures sensitive to both viruses. This agent, named RIF for resistance inducing factor, multiplied extensively in infected cultures, and could be assayed by an end point method. It was sedimented in the ultracentrifuge under conditions adequate to sediment RSV, and it could be neutralized by antiserum to RSV. These characteristics plus its widespread occurrence in eggs suggested that RIF is a virus of the avian lymphomatosis complex. Support for this relationship was obtained in the finding that four strains of lymphomatosis virus mimicked the action of RIF *in vitro* by inducing resistance to RSV, without causing overt cytopathology.

RIF was found to occur in high concentrations in the tissues of apparently normal embryos before explantation, ruling out the possibility that its production was activated by *in vitro* cultivation. It is suggested that the system for the detection of RIF and lymphomatosis virus might be a useful model to guide attempts to detect viruses in spontaneous tumors of other species.

The skillful technical assistance rendered by Miss A. Cornelius and Mrs. L. Fanshier is gratefully acknowledged.

* Supported by American Cancer Grant (E-82) and U. S. Public Health Service (Grant C-4774).

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*IMMUNOLOGICAL RELATIONSHIPS OF MEASLES, DISTEMPER, AND RINDERPEST VIRUSES**

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Communicated by Wendell M. Stanley, June 6, 1960

Evidence has accumulated from various laboratories to show an immunological relationship between canine distemper and measles viruses.[1-5] Investigators in Africa and Europe have demonstrated an immunological relationship between viruses of rinderpest and canine distemper.[6-8] Studies reported here present further evidence to substantiate the existence of an antigenic relationship between measles and distemper, and also show the presence of distemper and measles antibodies in rinderpest immune-bovine sera. These results indicate that the viruses share similar antigenic components. Serum samples from various hosts with rinderpest, measles, and distemper were analyzed for measles antibody by neutralization tests in tissue culture and for distemper antibody by neutralization tests in suckling mice.

Materials and Methods.—Serum samples: The normal and antirinderpest bovine sera were received from France. The antisera were prepared either with bovine rinderpest virus or with rabbit adapted rinderpest virus (strain Nakamura III). Normal serum samples were obtained from animals which were neither inoculated nor infected with rinderpest virus. Acute and convalescent sera were obtained from 10 children with clinically diagnosed measles.

Viruses: The Edmonston strain[9] of measles virus, propagated in HeLa cell cultures,[2] was employed for neutralization tests. The canine distemper strain was the mouse-adapted virus developed from the Lederle strain of avianized distemper virus[10] by successive serial brain passages in suckling mice.[2]

Neutralization tests: Measles neutralization tests were carried out in HeLa cell cultures as described in a previous paper.[2] Distemper neutralization tests were performed by mixing approximately 100 LD_{50} of mouse-adapted distemper virus with equal amounts of diluted test sera. Mixtures were incubated at 37°C for $^1/_2$ hr and placed in the refrigerator for an additional $^1/_2$ hr before inoculating 0.02 ml