by the as yet unresolved possible role of viral oncogenesis, that of Schwartz and Beldotti (*5*) was complicated by the frequent occurrence of transient mild-to-severe runting, which would indicate considerable injury to the lymphoid system. The studies are thus complementary, one obviating the runting syndrome and the other probably obviating viral oncogenesis. Together they would seem to present the strongest experimental evidence yet available that reactions intimately concerned with histocompatibility difference might play a role in the genesis of lymphomas.

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27 December 1965

Interferon: Lack of Detectable Uptake by Cells

Abstract. *No interferon, or at most a small fraction of that applied, is taken up by cells during the period of induction of antiviral activity. The lack of detectable adsorption was not influenced either by the concentration of interferon or the volume. The results were similar in both chicken and mouse systems.*

Many of the concepts of the mechanism of antiviral action of interferon are based on the assumption that cells rapidly adsorb the major portion of the applied interferon (*1*, *2*). In view of experiments which did not show disappearance of interferon from fluid, we have investigated the reaction of interferon with cells. Our results do not support the suggestion that interferon is rapidly adsorbed by cells, but they indicate that interferon can render cells resistant to virus in the absence of detectable adsorption.

Chick and mouse embryo tissue cultures and vesicular stomatitis virus were prepared, as described (*3*). Interferons from mouse serum and from chicken allantoic fluid were produced, characterized, and assayed (*3*). In the interferon transfer experiments, tissue culture plates were thoroughly drained by aspiration with capillary pipettes. This technique of thorough aspiration was also used to transfer interferon from one group of plates to another. Appropriate controls demonstrated that dilution by residual fluid on plates, evaporation during incubation, and the experimental procedure itself had no significant influence on the results.

In the first experiment, the conditions were similar to those reported for studies of interferon adsorption (*1*). Interferon (1.7 units) in 0.1-ml volume was added to ten thoroughly drained plates of chick embryo culture and incubated at 37°C. After 30 minutes, the interferon from five plates was recovered and assayed for residual activity. The interferon from the remaining five plates was recovered at 60 minutes and assayed (Fig. 1). The slope was determined by statistical analysis and indicates no significant decrease in residual interferon activity after a 1-hour incubation period with cells. For comparison, the slope of the reported consumption rate of 90 percent per hour is also shown (*1*).

If a subdetectable amount of interferon is actually taken up by cells then it might be detected if there was an increase in the number of cells exposed to the same amount of interferon. Such an increased ratio of cells to interferon was achieved by serial transfer of interferon onto cell cultures as follows. Chick interferon (30 units per milliliter) was incubated with chick embryo cell cultures for 3 hours, and then the culture fluid containing residual interferon was recovered. A portion of the recovered fluid was stored for later assay of interferon content, and the remaining fluid was transferred to new cell cultures. This was repeated for a total of eight serial transfers. In this way, the quantity of interferon adsorbed in 3 hours should be magnified eightfold. Three hours was sufficient to stimulate maximum antiviral activity in the plaque-reduction assay. Figure 2 shows the residual interferon titer as a function of the transfer number. The slope of the line as well as its 95 percent confidence limit is shown. The results

Table 1. Effect of serial transfer of 0.5 and 2.0 units (U) of mouse interferon on residual antiviral activity. Percentages determined from the mean value of five to eight plates per point.

Transfer No.	Cumulative (hr)	Inhibition of plaques by 0.5 U (%)	Inhibition of plaques by 2.0 U (%)
1	3	34	66
2	6	10	53
3	9	32	59
4	12	42	59

indicate that 0 ± 7.2 percent of the interferon was taken up by the cells during each transfer. Also shown are slopes representing a theoretical 50 percent uptake per passage and a theoretical 90 percent uptake per passage, which can be ruled out by comparison of the observed residual titer. Within the limits of these assays, it is not possible to detect any change in the amount of residual interferon remaining after eight serial adsorptions. If the uptake of interferon by cells were only 1 or 2 units per passage, then, in experi-

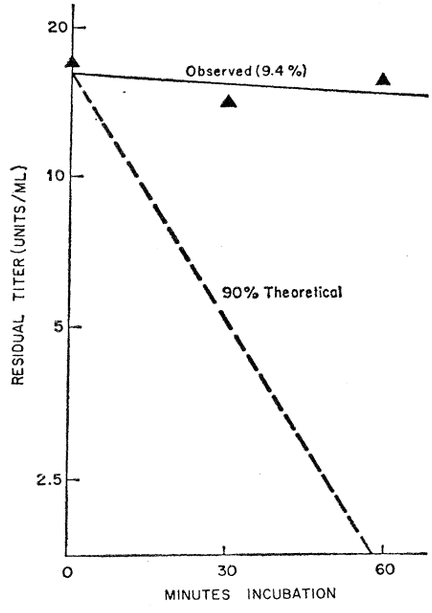

Fig. 1. Residual titer of a preparation of chicken interferon after incubation with chicken embryo cell cultures. Plaque counts from dilution assays of incubated preparations by the method of regression analysis (*12*) were found to be linear functions (with a common slope) of the log dilutions. Residual titers (50 percent inhibitory dilutions) were calculated from the individual regressions and the logarithms plotted as a function of the incubation time. The slope (b) of the line fitted to these values by least squares was expressed as: consumption (%)/hr = 100 ($1 = 10^b$).

ments where high concentrations are used, this amount of reduction might not be detected by the assay system used. To meet this objection, 0.5 and 2 units of mouse interferon in 1 ml were incubated for 3 hours with groups of five or more plates of mouse embryo cells for four serial transfers. After each adsorption period the group of treated cells were challenged with virus, and the percentage of inhibition was determined (Table 1). There was no significant change in the percentage of plaque inhibition during four serial transfers, indicating no loss of interferon activity. Similar results were obtained with 0.5 units of chicken interferon in 0.1-ml volumes on chick embryo cells.

Thus no interferon or, at most, a nondetectable, small fraction of the applied interferon is taken up by cells during the period of the induction of antiviral activity. This lack of adsorption was independent of the concentration of interferon or the volume applied. Similar results were obtained in both chicken and mouse systems in our laboratory and by others (*4*).

It seems unlikely that the production of new interferon by interferon-treated cells could have masked the uptake of significant quantities of the interferon originally applied, since its production has not been detected in cultures previously treated with interferon in the absence of virus challenge (*5*). Data obtained by rapid serial passage of tritium-labeled proteins of molecular weight in excess of 50,000 is consistent with the possibility that the very slight suggestion of adsorption of interferon might be attributable to dilution by residual fluids on the drained plates. Labeled cell proteins having a molecular weight between 20,000 and 30,000—compatible with that estimated for chicken interferon (*9*)—were found not to be adsorbed by the cells (*6*).

The uptake of interferon by cells cannot account for the rapid disappearance of interferon in vivo (*7*). Possible reasons for this disappearance are metabolic degradation and sequestration by the cells, but the dispersed interferon subsequently may be released by appropriate stimulation (*8*). Our results and those of Youngner and Stinebring (*4*) differ from previous reports of uptake of interferon (*1*, *2*). Possible explanations for most of the previous reports are nonspecific adsorption of interferon to containers and

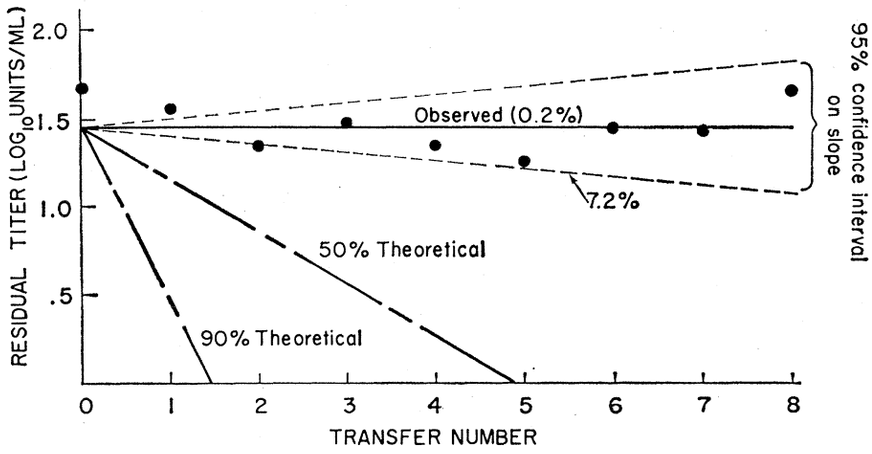

Fig. 2. Residual titer of a preparation of chicken interferon after eight, serial, 3-hour incubations on chick embryo cell cultures. The statistical procedure was applied to assays of the serially transferred preparations, the slope of the fitted line being expressed as percentage consumption per transfer. The confidence interval on this slope was calculated from the estimated residual titers.

cells, inactivation, and variability of the assay systems (*9*). The reported uptake of more than 90 percent of the applied chicken interferon by chick embryo cells (*1*) is more difficult to explain. The presence of live virus in the interferon preparation used complicates the interpretation of the results obtained.

Since the concentration of interferon outside the cells does not decrease with time of incubation, it is difficult to understand how one unit of interferon can inhibit only 50 percent of virus plaques regardless of the time of contact. It would seem that the undiminished presence of interferon should allow increasing protection after continued incubation. However, increased protection of the cells does not occur. This stabilized antiviral activity of cells, in the presence of interferon, could result from conditions such as (i) an equilibrium between continued induction of antiviral activity by interferon and an equally rapid loss of antiviral activity (*10*), and (ii) a mechanism that causes the interferon-treated cell to become refractory to continued effects of interferon.

Lack of detectable uptake of interferon requires a reexamination of the mechanisms by which interferon induces its antiviral effect. There are, at least, two possible ways in which interferon could react with cells to produce an antiviral effect. (i) It could act in a catalytic manner, not being consumed, but stimulating the production of another substance by the cell which is the actual antiviral substance, or (ii) it could enter into a combination with cell components to produce the antiviral state. Current estimates for the specific activity of interferon give values of about 4500 molecules per cell per unit of antiviral activity (*9*). Our results indicate that only a small fraction, at most, of the applied interferon could be consumed by cells. Synthesis of additional proteins would amplify the effect of these few molecules of interferon. Both of these pathways are compatible with the reported necessity for new synthesis of cell protein before interferon-treated cells become resistant to virus infection (*11*).

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Morphogenetic Substance in Legume Nodule Formation

Abstract. *Addition of extracts of bean cotyledon or hypocotyl or of a coconut-water preparation to roots grown from excised bean hypocotyls markedly increased the number of nodules, while removal of part of the hypocotyl tissue reduced the number. The active morphogenetic substance in the coconut water has been partially purified.*

Ability of plants to nodulate and to enter into a N_2-fixing symbiosis with species of *Rhizobium* is restricted to members of the Leguminosae. This fact suggests existence in the host of substances that govern the limited range of plants that the bacteria can invade and with which the microsymbionts can develop a functional relation. Moreover, the specificity of bacterium for plant, the morphological changes preceding infection, the alterations in root-hair structure during development of the infection thread, and the morphological changes associated with nodule genesis undoubtedly involve substances excreted by or contained within cells of one or both symbionts. There is also evidence of host control of nodule abundance, a control possibly implicating a morphogenetic substance synthesized in the green part of the plant and translocated to the root (*1*).

This report concerns the existence of morphogenetic substances implicated in nodule formation, and the development of a reliable bioassay for these substances. Our technique is based on a procedure developed by Raggio, Raggio, and Torrey (*2*), who demonstrated that roots excised from legumes will nodulate adequately in culture. This excised-root technique has produced direct evidence that a host factor is concerned in nodule genesis (*3*); and it was observed that the frequency of nodules is increased if the excised roots retain a portion of the hypocotyl (*4*).

Seeds of *Phaseolus vulgaris* L. were surface sterilized and soaked in water for 24 hours, and the axes below the cotyledons were excised—each piece about 6 mm long. The segments of excised tissue were incubated for 9 days at 22° to 23°C on White's medium (*5*), by which time they consisted of about 2 cm of hypocotyl tissue, primary root, and a few short adventitious roots. These structures were transferred to the split medium of Raggio *et al.* (*2*) containing 10 percent sucrose. The cut end of the plant material was inserted into a small glass vial containing nitrate, organic components of the autoclaved medium, and substances whose activity was to be tested. After 10 more days of incubation, when vigorous growth of the adventitious roots had occurred on the inorganic portion of the solidified medium, the roots were inoculated with a dilute suspension of *Rhizobium phaseoli*. Nodule counts and root measurements were made 3 or 4 weeks later; usually, a randomized complete-block design was employed; nodule numbers were statistically analyzed by use of a square-root $(x + \frac{1}{2})$ transformation. All attempts to culture the roots of this bean variety, detached from the hypocotyl, failed.

Table 1. Effect of coconut-water dialyzate on nodulation and root development by axes initially 6 mm long. In the analysis for statistical significance, the means from the 12 experiments were paired.

Nodules (mean No.)	Nodulation frequency (%)	Major roots, length (cm)	Lateral roots (No.)
	Untreated tissue		
10.5	90.5	45.9	29.1
	Treated tissue		
17.5	90.6	38.8	39.5
	Least significant difference (P=*0.1*)		
6.7	13.5	6.2	6.3

Table 2. Nodulation and growth of roots borne on segments of hypocotyls. Each treatment was replicated eight or nine times. Statistical significance between the untreated control and treated segments is shown by letters in parentheses: *N*, no significant difference at $P = .1$; *A*, *B*, *C*, significant differences at $P = .1$, .05, and .01, respectively. In each instance the frequency of nodulation was 100 percent. Treatment was with dialyzate of coconut water (CW) or of cotyledon extract (CE).

Treatment	Nodules (mean No.)	Major roots, length (cm)	Lateral roots (No.)
	Segments 10 mm long		
None	3.9	35	28
CW	13.9 (*C*)	37 (*N*)	29 (*N*)
CE	12.1 (*C*)	26 (*A*)	12 (*B*)
	Segments 15 mm long		
None	7.1	36	10
CW	13.7 (*B*)	26 (*A*)	18 (*N*)
CE	11.0 (*N*)	27 (*A*)	16 (*N*)

Table 1 summarizes the results of 12 experiments designed to determine the effect on nodulation of the dialyzate of autoclaved coconut water (30 percent by volume). Each tabulated value of nodule number and frequency and of root length and number represents the average of the means of observations from the 12 separate experiments, each containing seven to ten replicates. Although stimulation by the coconut-water preparation is unmistakable and statistically significant when all experiments are considered together, many nodules appeared even in the absence of the coconut water, making the enhancement of nodulation by the addition more difficult to evaluate. Consequently, possible modifications of the bioassay were studied that would permit more ready identification of substances implicated in nodule genesis.

The influence of the hypocotyl tissue was assessed by removing 10- or 15-mm segments of the hypocotyl from the structures developed from 6-mm axes. Roots developing from the remaining portions bore fewer nodules than the complete structures, yet no adverse effect on root growth was observed. The dialyzate from the autoclaved coconut water (provided in the organic medium at a concentration 30 percent of that in the original endosperm) trebled the numbers of nodules on the roots growing from the shorter structures, but the addition did not significantly influence root extension.

The coconut water had a similar effect when the 10- and 15-mm segments of hypocotyl tissue, cut from the complete structures, were employed; in addition, the dialyzate derived from the cotyledon extract (provided in the organic medium at a concentration equivalent to one cotyledon per milliliter) highly significantly increased the abundance of nodules ap-