ity in older rabbits between 4-10 months of age increases only slightly during 11 days of starvation. In the younger 30-75-day-old rabbits, intraperitoneal injection of 0.5 g L-tryptophan per kg body weight induces the enzyme between 7- to 15-fold over control values as compared to about 2-fold for 4-10-month-old animals.

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Inhibition of TRIC Agents by Virus-Induced Interferon.* (31150)

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The causative agents of trachoma and inclusion conjunctivitis (TRIC) have been called viruses in the past. However, TRIC agents possess several characteristics which clearly separate them from typical viruses. They possess a bacteria-like cell wall containing muramic acid; they contain both DNA and RNA; they elaborate enzymes involved in decarboxylation of carbohydrates and in folic acid synthesis; they are inhibited by antibacterial drugs; and at least some stages in the development of TRIC agents involve binary fission(1). Thus it appears that TRIC agents are more closely related to obligate intracellular bacteria or rickettsiae than to true viruses.

Up to the present time, interferons have been known to act only on true viruses. It was, therefore, of interest to determine whether virus-induced interferons would inhibit the replication of the more complex TRIC agents. Early in this work we were made aware of a publication by Mordhorst and Reinecke(2) in which it was claimed that interferon had no effect on TRIC agents. We wish to report here experiments which establish the inhibition of a TRIC agent by virus-induced mouse interferon in L cells.

Materials and methods. Interferon. Mouse interferon for these experiments was prepared by infecting strain L 929 cells, obtained from Dr. J. Youngner, with Newcastle disease virus (NDV) (E4 Herts strain) at a multiplicity of 1.5 pfu/cell (plaque forming units) as measured on chick embryo fibroblasts. The L cells were grown in one liter Blake bottles in a 5% CO_2 incubator at 37°C, using a growth medium of 10% calf serum in Eagle's Minimum Essential Medium (MEM) containing penicillin, streptomycin and mycostatin. Following infection, the cells were maintained on serum free MEM + antibiotics. Supernatants were collected 24 hours after infection. They were adjusted to pH 2 with 0.1 N HCl and held for 5 days at 4°C before neutralization with 0.1 N NaOH.

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When assayed against bovine vesicular stomatitis virus (VSV) in a plaque inhibition assay described elsewhere(3), this pool contained 2,000 units of interferon activity per ml. Employing gradient chromatography on the ion exchange resin XE-64(3), partially purified mouse interferon was prepared from a portion of this pool.

For characterization of the inhibitory factor present in the mouse interferon preparation, one aliquot was adjusted to pH 2 with 0.1 N HCl, maintained at 4°C overnight, and brought back to neutrality with 0.1 N NaOH before transfer to cell monolayers. An equal volume of MEM was treated similarly. Another aliquot was spun at 100,000 *g* for one hour. The upper half of the supernatant fluid was removed, and the pellet was resuspended in the lower half of the fluid, in order to determine sedimentability. In another experiment, mouse interferon diluted 1:5 was added to tubes containing trypsin (0.25 mg/ml) or to an equal volume of MEM; trypsin was also added to MEM in a third tube. These 3 tubes were then incubated at 38°C for one hour before inoculation onto monolayers.

Infective agent. The LB-1 strain (TRIC/ /GB/MRC-1/G) of inclusion conjunctivitis agent(4) was obtained from Dr. L. H. Collier in its forty-second yolk sac passage. It was passed in eggs in our laboratory, and a pool of infected yolk sacs from the forty-eighth passage served as inoculum for the series of experiments to be reported here. The methods of preparing and storing stock pools of infective material and their assay have been described(5). Strain LB-1 proliferates readily in cell culture(6) and forms easily identifiable intra-cytoplasmic inclusions within 48 hours of inoculation. However, at 48 hours only a single cycle of infection will take place with no secondary spread of the infecting agent to adjoining cells.

Tissue culture. Strain L 929 cells originally obtained from Dr. T. Akers were used throughout the study. Stocks were grown as stationary cultures in 4 oz prescription bottles with a medium containing 10% fetal calf serum, 0.5% lactalbumin hydrolysate, 0.1% yeast extract, 0.5% glucose in Mixture 199 and Hanks' BSS, with 300 μg/ml penicillin and 500 μg/ml streptomycin. For the experimental studies cells were trypsinized (0.25%), resuspended in the medium described above but without antibiotics, approximately 25,000 cells in 1 ml volume seeded into Leighton tubes containing coverslips, and incubated at 35°C. At 24 or 48 hours the growth medium was removed and 1 ml of the various interferon preparations was added. The cells were incubated at 35°C for 6 hours, the fluid was removed, the cell sheets washed 3 times with Hanks' BSS, and the infective inoculum of LB-1 added. After 2½ hours at 35°C the inoculum was removed, the cells washed twice with Hanks' and 1 ml of a medium similar to that described above, but with the glucose concentration increased to 2%, was added to each tube.

Quantitation of TRIC agent. Count of inclusion-forming units (ifu). Coverslips were removed from Leighton tubes 48 hours after addition of the LB-1 inoculum, washed in saline, and drained. They were then transferred to absolute methyl alcohol at room temperature for one hour or more, stained with 10% Giemsa for 30 minutes, rinsed in tap water, dried, rinsed briefly in xylene, and mounted. The slides were examined at a magnification of 1200× with the oil immersion objective. The number of typical stained inclusions per hundred cells was counted on triplicate slides by 2 observers. There was always close agreement among counts from triplicate slides.

The methods for determining ELD50 and EID50 of the yolk sac material have been described(5). In addition, the total number of particles was estimated by direct count (7). Yolk sac material was treated with trypsin, centrifuged, and resuspended. Particles were stained with Giemsa and examined by darkfield microscopy with the oil immersion lens(7).

Results. Initially, L cell monolayers were infected with a dilution of LB-1 pool containing per ml approximately $10^{8.3}$ particles and $10^{6.1}$ EID50. Monolayers pretreated with Eagle's MEM yielded 78 ifu/100 cells, whereas monolayers pretreated with undi-

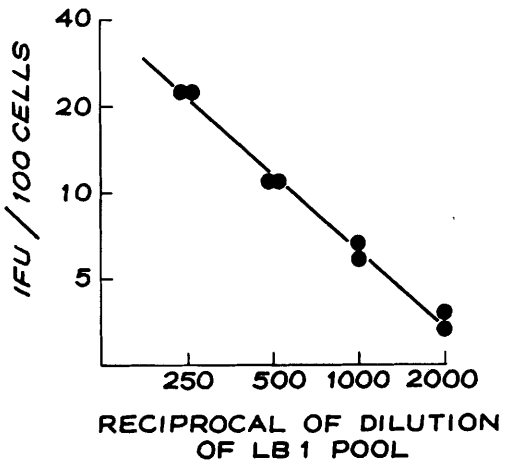

FIG. 1. Relationship between dilution and number of inclusion-forming units of LB-1 in L cells.

luted mouse interferon yielded only 16 ifu/100 cells, a reduction of 80%.

To reduce the probable large excess of inactive particles in the above inoculum and avoid some possible autointerference, all subsequent experiments were performed with a 2×10^{-3} dilution of the LB-1 pool which contained $10^{7.3}$ particles/ml and $10^{5.1}$ EID-50/ml. In this range there was a direct relationship between ifu/100 cells and concentration of inoculum (Fig. 1). When 1 ml of this material was inoculated onto monolayers of approximately 50,000 cells, the input consisted of 2.5 EID50 or 400 particles per cell. This inoculum yielded regularly 26 to 33 ifu/100 cells in MEM treated controls (Fig. 2). Undiluted mouse interferon reduced the ifu yield by 90% (Fig. 3, Table I). A dose response curve for mouse interferon in this system is shown in Fig. 4. It appears that 50% inhibition is achieved by a 1:200 dilution of mouse interferon. Thus, mouse interferon contains 200 units when measured against TRIC agent compared to 2,000 units against VSV.

Table I lists the features of the TRIC inhibition which are quite typical of an interferon mediated effect(8). The inhibitory activity was not affected by exposure to pH 2, nor was it sedimented by 100,000 $\times$ g for one hour. It was destroyed by treatment with trypsin or by heating at 70°C for one hour. The effect was clearly species-specific: Mouse interferon was active against the agent in mouse L cells, but human interferon had no inhibitory effect in TRIC replication in the same cell line. This human interferon was prepared by NDV infection of human neonate fibroblasts as described elsewhere(9) and had an activity of 1:700 against VSV on human fibroblasts. In addition, the in-

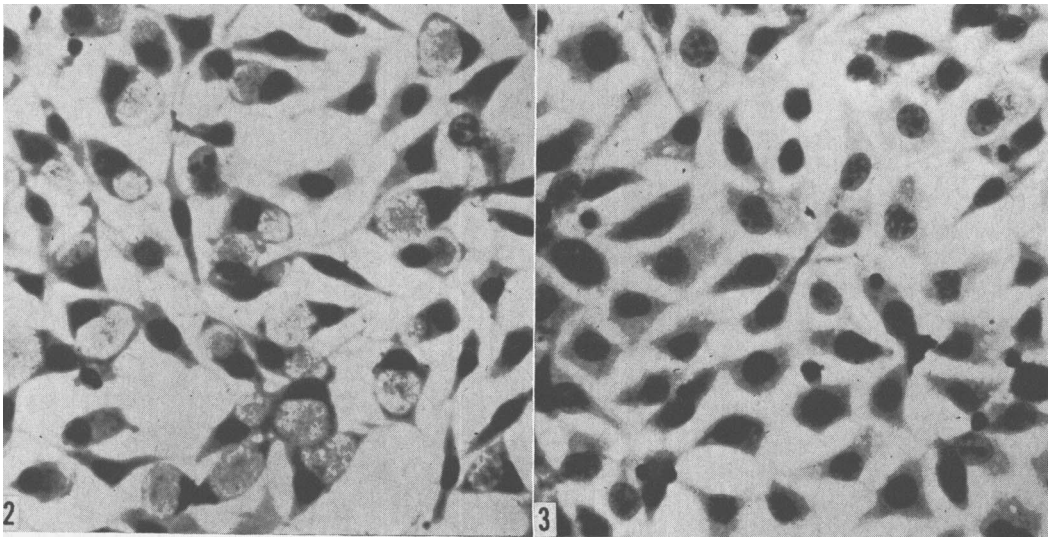

Fig. 2. L cells in culture infected with LB-1. Many cells show typical intracytoplasmic inclusion bodies filled with elementary bodies.

FIG. 3. L cells infected with same inoculum as in Fig. 2, but pretreated with mouse interferon diluted 1:5. Only one inclusion is seen in the field.

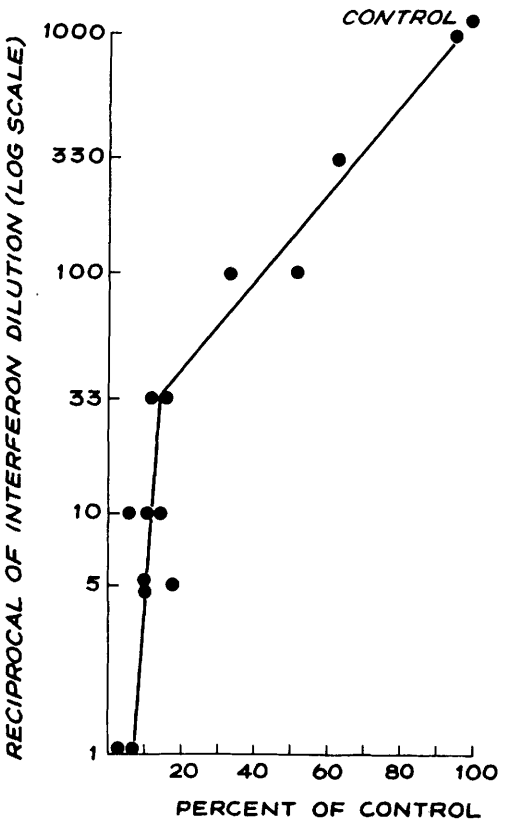

FIG. 4. Dose response curve of mouse interferon acting on LB-1 infection in L cells.

hibition could be demonstrated with mouse interferon purified by column chromatography.

To determine what effect the mouse interferon might have directly upon the TRIC agent, 0.2 ml of a 50% suspension of LB-1 was added to 0.8 ml of mouse interferon diluted 1:5 and to MEM; the mixtures were held at approximately 25°C for 4 hours, with occasional shaking. The material was then diluted 1:50, resulting in 1:500 final dilution of LB-1 and 1:250 dilution of interferon. This material was inoculated onto cell monolayers, allowed to adsorb for 2½ hours, and thereafter treated routinely. There was no significant difference between the average counts for the 2 preparations, indicating that the interferon had not affected the TRIC agent directly.

Discussion. Many complex molecules have been found capable of inducing cells to form interferon-like substances. The activity of such interferons was always assayed against viruses, and this effect specifically directed against viruses was believed a characteristic of interferon. It is not known what precise step in viral replication is blocked by interferons. However, the basic similarity of all biosynthetic patterns suggests that interferons may affect replicative processes in higher organisms as well. The present demonstration of the inhibition of TRIC agent replication by virus-induced interferons supports this belief. TRIC agents are probably more closely related to bacteria than to viruses, and by proper quantitative methods it may be possible to demonstrate interferon effects on the replication of intracellular bacteria.

This paper records the first quantitation of interferon effects on TRIC and related agents. The features of TRIC agent inhibition are those usually attributed to interferon. Sueltenfuss and Pollard(10) suggested that the inhibition of maturation of a psittacosis agent in cell culture could be used as an assay for interferon induced by duck hepatitis virus in chick cell culture. Their method did not permit good quantitation of the interferon effect. Hopps *et al*(11) reported the

TABLE I. Effect of Various Interferon Preparations on L Cell Infection by TRIC Agent.

Treatment of L cell monolayer prior to infection with TRIC agent (26-33 ifu/100 cells)			ifu % yield
Maintenance medium MEM			100.0
MEM treated at pH 2 for 16 hr, then neutralized			100.0
Mouse interferon		crude 1:1	7.7
"	"	chromatographed, purified, 1:2	18.5
"	"	1:5 treated at pH 2 for 16 hr, then neutralized	9.1
"	"	1:5 heated 38°C for 1 hr	9.1
"	"	1:5 heated 57°C for 1 hr	30.3
"	"	1:5 heated 70°C for 1 hr	78.8
Human	"	crude 1:1	85.2
Mouse	"	1:5 centrifuged 100,000 × *g* for 1 hr	
		top half	9.1
		bottom half	7.7
MEM treated with trypsin 0.25%, 1 hr, 38°C			100.0
Mouse interferon, 1:5, treated with trypsin 0.25%, 1 hr, 38°C			96.0

production of interferon-like activity in cell cultures infected with a rickettsia but did not describe the inhibition of rickettsial multiplication by this substance. In the accompanying paper(12) the characteristics of interferon induced by a TRIC agent are detailed.

Mordhorst's(2) failure to demonstrate susceptibility of TRIC agent to interferon can be attributed in part to the lack of an adequate quantitative method and in part to the use of a low potency interferon. In our studies the activity of mouse interferon was 10-fold lower against TRIC agent than against VSV titrated in the same cell strain. The reason for this lower susceptibility needs to be further explored.

TRIC agents produce protracted infections in the human eye. It cannot be stated what role, if any, locally produced interferon might have on the replication and persistence of the infectious agent. The present documentation of TRIC agent inhibition by interferon may play no practical role in disease but has important implications regarding the broad nature of interferon activity.

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Characteristics of Interferon Induced *in vitro* and *in vivo* by a TRIC Agent.* (31151)

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Interferon has been induced by virtually all types of animal viruses in a number of mammalian cell species, both *in vivo* and in tissue culture(1). More recently, a number of nonviral agents have induced interferon production, including *Rickettsia tsutsugamushi*(2), brucella(3), pleuro-pneumonia-like organisms (Stinebring *et al*, personal communication), endotoxin(4), phytohemagglutinin(5), and statalon(6).

One attempt to demonstrate chick interferon production with trachoma-inclusion conjunctivitis (TRIC) agents *in ovo* and *in vivo* resulted in failure(7). These agents differ from true viruses in many important aspects of their structure and life cycle and, like the other members of the psittacosis-LGV-trachoma group, resemble rickettsiae and bacteria(8). Although nonviral agents induce interferon in serum which apparently has somewhat different physical characteristics compared to virus-induced interferon(9, 10), we thought it surprising if the TRIC agents lacked an interferon-inducing capacity. Therefore, appropriate experiments were performed in which interferon induction was successfully demonstrated.

Materials and methods. Interferon assay. A highly interferon-sensitive line of L cells obtained from Dr. J. Youngner was employed in these studies. These L cells are approximately 10 times more sensitive than mouse

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