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Toxoplasma gondii: Human Interferon Studies by Plaque Assay (40588)[1]

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Interferons and their inducers affect the replication and growth of numerous microorganisms in a variety of cells and biological systems (1, 2). Although much progress has been made in understanding the mechanism(s) of antiviral activity of interferon (IF), interactions of the IF system with more complex organisms such as intracellular bacteria, chlamydia, and protozoa remain unexplained (3, 4).

Toxoplasma gondii (TG), a protozoan capable of infecting a broad range of animal cells (5), seems to be both sensitive to and an inducer of IF in chick and mouse systems. Rytel and Jones detected the appearance of a viral inhibitor compatible with IF in the sera of mice infected with TG (6). Freshman *et al.* found a similar inhibitor in TG-infected mouse peritoneal exudate (7). Remington and Merigan showed mouse and chick IF to inhibit TG growth in homologous monolayers (8). Demonstration of similar properties in human-derived systems would have important theoretical and perhaps practical implications.

Taking advantage of the plaque-forming ability of TG in cell monolayers (9) and the suitability of viral plaque-reduction assays for IF studies (1), a TG plaque assay was devised to study the effects of human IF on TG *in vitro*. The induction of IF activity by TG in human cells was also examined.

Materials and methods. (i) Plaque formation by Toxoplasma gondii. Diploid human embryonic fibroblasts (HEF) were grown to confluence in 2 cm^2 wells of 24-well tissue culture plates (FB-16-24-TC; Linbro Chemical Co., New Haven). Monolayers were maintained under bicarbonate-buffered Eagle's basal medium with 50 units penicillin and 50 μg streptomycin per ml and 2% heat-inactivated fetal calf serum (BME/2), at 37° in humidified air with 5% CO_2.

RH-strain TG was passaged intraperitoneally in BALB/c mice. On the third day, fresh peritoneal exudate contained 10^7–10^8 trophozoites/ml, with no more than one murine cell per 10^2–10^3 trophozoites. Suspensions were diluted in BME/2 to a final concentration of 50–200 refractile and motile trophozoites per ml.

HEF monolayers were rinsed, inoculated with 0.2 ml of TG suspension, incubated for 2 hr for adsorption, rinsed, and overlaid with fresh BME/2 containing 1% methylcellulose. After 5 days further incubation, the overlay was aspirated and the wells rinsed, fixed with formalin, and stained with ethanolic crystal violet. Plaques (Fig. 1) were counted using a dissecting microscope or a microfilm reader (Fig. 2). The number of plaques was consistently within 20% of the number expected on the basis of hemocytometer counts of the inoculum, using refractility and motility as criteria for presumed viability of trophozoites.

(ii) Interferon assay. Confluent 2 cm^2 HEF monolayers were prepared as above. Following 24-h incubation with serial half-log_{10} dilutions of the material being assayed, monolayers were rinsed and inoculated with 0.2 ml of BME/2 containing 10–40 plaque-forming units (pfu) of Vesicular Stomatitis Virus (VSV, Indiana Strain), reincubated for 2 hr, rinsed, overlaid as above, reincubated for 36–48 hr and finally fixed, stained, and examined as above. All IF assays were performed in parallel with dilutions of $(I \cdot C)_n$-induced human fibroblast IF (initial titer 2–3 $\times$ 10^5 reference units/ml) or Sendai virus-induced human leukocyte IF (10^4 units/ml).

(iii) Interferon induction. HEF were grown to confluence in 25-cm^2 flasks and maintained for 1 week before use. Following rins-

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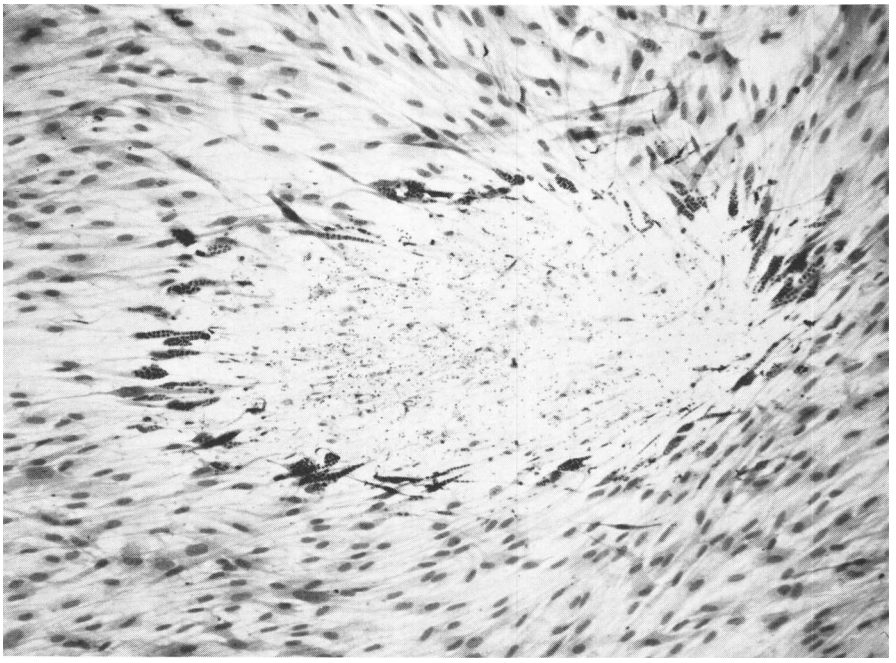

FIG. 1. Characteristic *Toxoplasma gondii* plaque in monolayer of human embryonic fibroblasts, 3 days after inoculation. Plaque consists of cell debris and free organisms, rimmed by infected cells. × 100 (H & E).

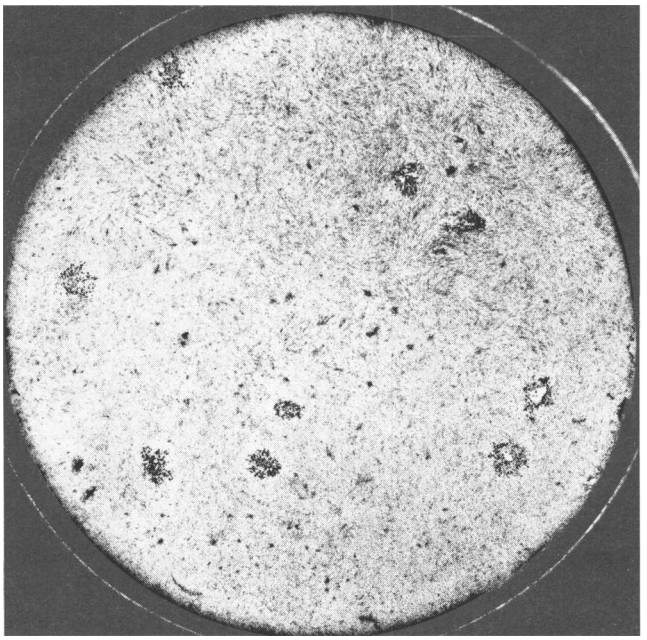

FIG. 2. The 2-cm^2 monolayer of human embyronic fibroblasts in cell-culture well, as utilized for plaque assay of *Toxoplasma gondii.* Nine TG plaques are clearly seen, with two more partly visible at the right margin. Methylcellulose overlay, 5 days incubation, formalin fixation, and ethanol-crystal violet staining are as described in text. × 6.

ing, monolayers were inoculated with either 2×10^6 viable RH TG, or 10^8 pfu of Newcastle Disease Virus (NDV, Herts Strain) in 1 ml BME/2, incubated for 2 hr, rinsed, and reincubated with 3 ml BME/2 for 24 hr. The medium was removed, clarified by centrifugation, acidified to pH 2 with HCl, kept at 4° for 5 days, adjusted to pH 7 with $NaHCO_3$, irradiated with ultraviolet (uv, General Electric GT-85 germicidal lamp) with a measured flux of 1.5×10^7 ergs/ml at 2537 Å (10), and stored at −20° until assayed for IF.

(iv) Interferon induction in human peripheral leukocytes. Two healthy adult donors were studied. One had no history suggestive of prior toxoplasmosis and had dye and indirect fluorescent antibody titers <1:16. The second had laboratory-acquired toxoplasmosis 20 years earlier (11), and has maintained a dye titer of 1:256 while continuing to work with TG. Peripheral mononuclear cells were prepared from fresh heparinized blood by standard methods using a Ficoll–Isopaque gradient (12) and dispensed into glass screwcap tubes in aliquots of 1.4×10^6 cells in 0.5 ml BME/2. One ml BME/2 containing either 10^7 RH TG, 5×10^7 pfu NDV, or no organisms was added to each tube; after 2-hr stationary incubation the tubes were centrifuged, the supernatants replaced with 1.5 ml fresh BME/2, and the tubes reincubated for 24 hr. The medium was then removed from each tube, acidified and irradiated with uv as described above, and assayed for IF activity.

Results. (i) Susceptibility of RH toxoplasma to interferon. Monolayers of HEF were pretreated for 24 hr with dilutions of fibroblast or leukocyte IF in concentrations ranging from 10^{-1} to 10^4 reference units/ml, before infection with approximately 30 viable plaque-forming RH TG or 10 pfu VSV. VSV plaque numbers were reduced 50% at 2.4 units/ml of fibroblast IF and at 10 units/ml of leukocyte IF. There was no effect on TG plaque numbers or size at any IF concentration tested, as compared with control wells.

(ii) Interferon induction by RH toxoplasma. No IF activity was detected in the supernatants from cultures infected with TG, as manifested by plaque-reduction of VSV, even when assayed without pretreatment by acid or uv. Fluids from HEF infected with NDV contained acid- and uv-stable IF activity at a titer of 1:365, equivalent to 900 reference units/ml; the calculated specific activity of this material was 2700 units/10^6 cells, or 2.7×10^{-3} unit/cell, in the VSV plaque-reduction assay. These fluids had no effect on TG plaques at any concentration.

In an attempt to enhance IF induction by pretreatment of monolayers with uv (13), 60-mm petri dish monolayers of HEF (approximately 8×10^5 cells each), 7 days postconfluence, received 660 ergs/mm^2 of uv immediately preceding inoculation with 1.5 ml BME/2 containing 2×10^7 viable TG. Following 2 hr incubation, the cells were rinsed and reincubated under 4 ml BME/2 for 24 hr prior to IF assay of the medium. Again, no IF activity was detected.

(iii) Interferon induction in human peripheral leukocytes. Suspensions of peripheral lymphocytes and monocytes from two donors, one with a past history of toxoplasmosis, showed no differences in production of acid- and uv-stable IF: similar levels following induction with NDV, approximately 35–100 reference units/ml (3.5 to 10×10^{-5} unit/cell); no detectable IF activity after TG challenge when assayed by VSV plaque reduction; and no activity against TG plaques.

Discussion. Interferon activity can be demonstrated by a variety of *in vitro* techniques, some of which have been applied to prior studies with TG. Chaparas and Schlesinger reported the ability of TG to form discrete plaques in chick-cell monolayers (9); Remington and Merigan adapted a plaque-reduction assay, as well as yield-reduction and CPE-inhibition assays, to demonstrate protection of chick and mouse cell monolayers from destruction by TG when treated with IF (8). Schmunis *et al.* employed a yield-reduction assay in their study of the effects of $(I \cdot C)_n$ and mouse and rabbit IF on the growth of TG in several cell types—human conjunctiva and WI-38, rabbit kidney, and mouse L-929—with negative results (14). Studies of the effects of human IF on TG growth in human cells have not been published heretofore.

For this study, plaque reduction was selected for precision and sensitivity. The accuracy of yield-reduction is limited by the difficulty in controlling for organisms which are not released from unlysed cells, which

remain adherent to the substrate, which adhere to one another, or which may be nonviable or noninvasive. CPE inhibition depends on semi-quantitative and somewhat subjective evaluation of grossly visible changes; the presence of a few organisms in cells may be easily overlooked in the scanning of many cultures. TG plaques in human fibroblasts grow moderately slowly but are discrete, easily counted, and numerically reproducible.

The experiments showed that the plaque-forming ability of RH TG is highly efficient and not inhibited by high concentrations of human IF. There were no apparent differences in time of appearance, rate of growth, numbers of cells infected, or density of intracellular parasites at plaque rims between TG plaques formed in the absence of IF and those formed in the presence of all tested concentrations of IF. Although more subtle effects of IF on TG multiplication could not be directly demonstrated, it would appear that human IF has no influence on the *in vitro* growth of TG in human cells.

Is TG an inducer of IF in human cells? The test system in these experiments responds well to viral induction, with IF production comparable to values computed from other reports (15, 16). By contrast, no IF was detected following infection by TG. It seems reasonable to accept the finding that TG is at least two to three $\log_{10}$ less potent than NDV as an inducer of IF in these cells. The lack of any effect on TG growth of fluids from TG-infected cultures suggests furthermore the absence of a TG-specific IF-like system in human cells. The evaluation of other studies of IF induction by TG must take into account "immune IF" (17, 18) which may be produced along with other lymphokines; "immune IF" is acid labile, and was not searched for in these experiments. The production of acid-stable IF by TG infection *in vivo* (6, 7) may represent a species-specific phenomenon or, alternatively, interactions much more complex than a single-cell *in vitro* system can demonstrate.

Summary. In order to define the interaction of the human IF system with TG, a series of experiments examined the effects of human IF on TG growth in monolayers of HEF.

Neither leukocyte nor fibroblast human IF had any effect on TG plaque growth, in IF concentrations ranging from 10^{-1} to 10^{4} units/ml, although appropriate IF activity was demonstrated in VSV plaque-reduction assays performed in parallel. Supernatant fluids from TG-infected cells, both HEF and peripheral human leukocytes, contained no detectable acid-stable IF activity, despite good induction of IF by NDV in parallel experiments. Supernatants from TG-infected cells contained no inhibitor of TG as assayed with TG plaques in HEF.

These experiments indicate a lack of interaction between TG and the classical IF system in cells of human origin, supporting the findings of Schmunis *et al.* (14). It is clear that the observations in murine and chick models (6–8) cannot be directly extrapolated to humans, and that interactions of TG and the IF system are a function of the origin of the IF and of the cell system under study.

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