INTERFERON AND AN INTERFERON ANTAGONIST

Annie R. Beasley, et al

Miami University

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"INTERFERON AND AN INTERFERON ANTAGONIST"

Annie R. Beasley, Principal Investigator

M. Michael Sigel, Program Director

Department of Microbiology
University of Miami
School of Medicine
Miami, Florida 33152

I. Pursuit of a study of a mouse cell interferon antagonist effective in Ehrlich ascites tumor cells.

Previous workers in this laboratory (1) found crude Newcastle disease virus (NDV) - induced interferon produced in L-929 cell cultures to be highly effective in protecting these cells against Mengovirus but totally incapable of protecting mouse-passaged Ehrlich ascites tumor (EAT) cells. They found this anamolous intraspecies specifity to be due to the presence in the crude material of an interferon antagonist. Adsorption on CM Sephadex C25 and elution with a rising pH gradient effected isolation of semipurified interferon, fully protective of EAT cells, as well as separation of the antagonist. The latter eluted in two positions - one just after the loading volume and the other just prior to interferon elution. Our initial objective was to characterize the antagonist by biochemical and biological parameters.

L cells (NCTC clone 929) were obtained from the American Type Culture Collection. For interferon (IF) production, monolayer cultures in 8 oz. prescription bottles were inoculated with NDV and incubated 2 hours. Unadsorbed virus was removed, serum-free medium was added, and cultures were further incubated overnight. Pooled harvests of culture fluids were subjected to low speed centrifugation; supernates were acidified to pH 2.0, held at 4°C for 5 days, and subsequently neutralized. All such

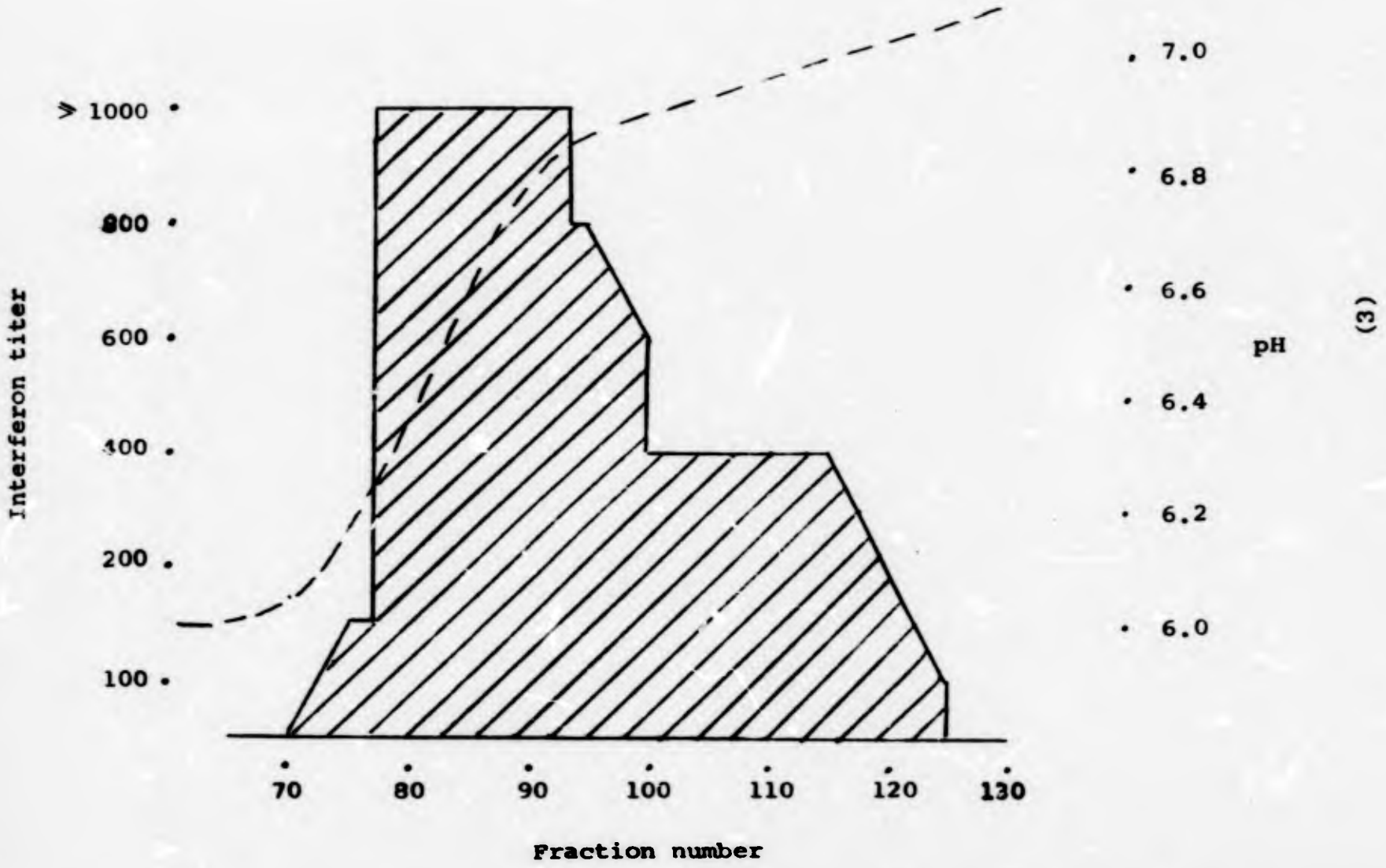
preparations had titers of 3200 units/ml or greater as determined by the inhibition of cytopathic effects (CPE) following Mengovirus challenge of pre-treated L cell monolayer cultures. That these inhibitory effects were not due to induction of IF in assay cultures by residual NDV was evidenced by the fact that ultracentrifugation did not diminish the antiviral activity.

Labelled IF was similarly obtained by incubation of NDV infected L cells in nutrient medium containing 1μc/ml of a protein labelling mixture of ^{14}C l-arginine, l-leucine, l-lysine and l-valine (Schwartz Bioresearch Inc.).

The crude IF was partially purified by adsorption to CM Sephadex C25 and subsequent elution with a rising pH gradient according to the method of Merigan et al. (2). Five ml fractions were collected. Aliquots were used for pH determination and calf serum to give a final concentration of 5% was added to the remaining portion of each fraction to stabilize the IF. Preparations were stored at -40°C. Various fractions were selected on the basis of their pH and assayed in L cells for IF content. A typical elution profile is shown in figure 1.

IF produced in the presence of the labelled amino acids was exhaustively dialyzed against phosphate buffered saline and partially purified as above. Aliquots of alternate fractions were assayed for radioactivity in a Packard Tricarb liquid scintillation counter. Initial fractions (through No. 19) had relatively high counts, then there was a precipitous drop in activity, as

Figure 1. Elution of interferon from CM Sephadex C15



(3)

shown in Table 1; concommitant with elution of IF was an increase in radioactivity, as seen in Table 2.

The assay procedure of Truden et al. (1) for an IF antagonist was employed. For this purpose, mice bearing Ehrlich ascites tumor (EAT) cells were obtained from a local laboratory (Variety Children's Research Foundation). The tumor cells were subsequently maintained through serial mouse passage.

Test preparations consisted of eluates from the crude IF separation shown in figure 1, equal aliquots of every 10 consecutive fractions being pooled. The semipurified IF employed consisted of a pool of fractions 78 - 93 from the same separation. Equal volumes of the respective test preparations and of IF were mixed and mixtures were diluted 1:10 in special spinner medium (SSM). (This consisted of Eagle's basal medium in modified Hank's balanced salt solution - minus calcium and magnesium, phosphate reduced to 0.01 of the usual amount, and glucose replaced by galactose. It was supplemented with 5% dialyzed newborn calf serum and 0.02M Tris, pH 7.2, was incorporated as buffer.) To a 5 ml aliquot of each of the various mixtures was added 10^7 washed EAT cells as contained in 5 ml. Controls consisted of cells plus an appropriate dilution of IF alone and of cells plus SSM.

Mixtures were incubated at 37°C overnight in a roller drum and viable cell counts were made by the trypan blue dye exclusion technic. The cells were sedimented by centrifugation and washed with SSM. Each cell pellet was resuspended in a small volume of

Table 1. Radioactivity of eluates from CM Sephadex chromatography of labelled IF.

Fraction #	DPM/ml	Fraction #	DPM/ml
1	17	15	12,284
3	5,985	17	11,915
5	7,203	19	10,908
7	12,359	21	2,027
9	9,645	23	314
11	8,626	25	304
13	9,378		

Table 2. Radioactivity and IF titer of eluates from CM Sephadex chromatography of labelled IF.

Fraction #	DPM/ml	Units IF/ml*
75	20	<200
79	41	<200
83	171	<200
91	391	600
93	2664	600
96	2488	800
97	3102	800
105	4172	1000
111	3984	800
116	641	200

* One unit of IF is the least amount affording 50% protection of L cells against Mengovirus challenge as measured by inhibition of viral CPE.

SSM and infected with $10^{7.5}$ ID_{50} of Mengovirus. Mixtures were incubated at 37°C for 45 minutes, following which the cells were sedimented by centrifugation, washed once with SSM and finally resuspended in 10 ml SSM. Cultures were further incubated at 37°C for 24 hours then frozen.

Plaque assays for infectious virus were made in L cell monolayer cultures. Results are shown in Table 3. These results are obviously equivocal. Of particular concern were the high percentages of nonviable cells at the time of virus challenge. In addition, the virus control titer was much lower than anticipated and the interferon alone effected less than 50% reduction in virus replication. (It is to be reiterated that the IF contained 1000 or more units/ml when assayed in L cells by the CPE-inhibition technic.) If the results are significant, they suggest eluates in fraction pools A, C and F exerted a degree of antagonistic activity against IF.

These results prompted an assay of various IF preparations in spinner cultures of EAT cells. The test materials were (1) pooled semipurified IF from the above experiment, stored at -70°C since pooling, or (2) stored at 4°C since pooling; (3) a semipurified IF which had never been thawed; (4) crude IF; and (5) SSM to serve as virus-cell control.

Mixtures of test materials and of washed EAT cells were prepared to give final test preparation dilutions of 1:20 and 10^6 cells/ml in 20 ml volumes. Cultures were incubated overnight

Table 3. Assay of eluates from CM Sephadex chromatography of crude IF for IF antagonist.

No.	Culture Test preparation	% viable cells	Average PFO/ml Counted	Normalized*
A	IF + fractions 1-10	32.5	33.7×10^2	10.4×10^3
B	IF + fractions 11-20	32.9	25.3×10^2	7.7×10^3
C	IF + fractions 21-30	27.8	31.3×10^2	11.3×10^3
D	IF + fractions 31-40	35.7	34.3×10^2	9.7×10^3
E	IF + fractions 41-50	43.2	17.3×10^2	4.0×10^3
F	IF + fractions 51-60	26.2	30.3×10^2	11.5×10^3
G	IF + fractions 61-70	27.6	23.3×10^2	8.4×10^3
H	IF + SSM	34.9	26.7×10^2	7.7×10^3
I	SSM	26.7	32.0×10^2	12.0×10^3

* Corrected to 100% viable cells.

at 37°C with constant stirring. Viable cell counts were made, cells were sedimented by centrifugation and each cell pellet was resuspended in 4 ml SSM then infected with 0.8 ml Mengovirus ($10^{8.5}$ ID_{50}/ml) and incubated 45 minutes at 37°C. Cells were washed twice with 25 ml aliquots of balanced salt solution (BSS) then resuspended in 20 ml volumes of SSM. After incubation at 37°C for 24 hours with stirring, aliquots were stored at -70°C for subsequent plaque assays of infectivity in L cell cultures.

Results are shown in Table 4. Again, there was relatively little virus replication, although cell viability was improved. Crude IF was devoid of inhibitory activity as was the semipurified IF pool stored at -70°C. (The latter had been twice frozen and thawed.) Further, the single fraction of semipurified IF had less than 50% inhibitory effect - below that expected on the basis of titration in L cell cultures.

In consideration of the low level of virus replication in the above experiments, EAT - bearing mice were obtained from a different source (L. S. Dietrich, of this University). Cells from the ascitic fluids were washed and diluted in SSM to a concentration of 2 x 10^6/ml. Five ml aliquots were mixed with equal volumes of (a) undiluted Mengovirus, (b) Mengovirus diluted 10^{-1}, and (c) SSM. To another 5 ml of undiluted virus was added 5 ml of SSM. All mixtures were incubated at 37°C for 1 hour. To each was added 20 ml BSS and mixtures were centrifuged to sediment the cells.

Table 4. The effects of various IF preparations on the replication of Mengovirus in EAT cell suspension cultures.

Test interferon	% viable cells	Virus yield, PFU/ml
Semipurified, 2x freeze-thaw	72.9	7.3×10^2
Semipurified, stored 4°C	60.3	5.7×10^2
Semipurified, stored -40°C	72.4	4.1×10^2
Crude, stored 4°C	66.3	7.7×10^2
None (virus control)	62.3	7.7×10^2

Supernates were discarded and the "pellets" washed twice with 25 ml BSS and cells again sedimented. Each "pellet" was then resuspended in 5 ml SSM. Replicate 2 ml aliquots were placed in 12 x 100 mm tubes and incubated at 37°C with constant agitation by micro magnetic stirring bars. Additional aliquots were frozen for subsequent virus assay. After overnight incubation, 0.5 ml was removed from each infected culture and frozen, viable cell counts were determined for the cell controls, and incubation continued. One culture/ virus inoculum was frozen 24 hours post-infection and the other was frozen after a further 20 hour incubation. Infectivity assays were made in monolayer tube cultures of L cells, with the results shown in Table 5.

It is impossible to say whether virus replication occurred in these EAT cells under the experimental conditions employed, or even whether virus had adsorbed to the cells. Further, it is seen from the cell-free system that the washing procedure was inadequate to remove unadsorbed virus.

As a further investigation of this latter point, 1 ml aliquots of Mengovirus were mixed in 15 ml conical centrifuge tubes with equal volumes of BSS supplemented with 5% calf serum (BSS-5C). All were incubated at 37°C for 1 hr. After this period, the contents of 1 tube were frozen as "unwashed" virus. To each of the remaining tubes was added 8 ml of BSS-5C. All were centrifuged and supernates decanted. The residual fluids were mixed with 9 ml volumes of BSS-5C a procedure which was repeated a total of 8 times. At the end

Table 5. Replication of Mengovirus in mouse EAT cells in suspension culture.

Culture number	Incubation time*	Virus yield, ID_{50}/ml, inoculum: Undiluted	10^{-1}
Pool of I and II	1 hr.	$\geq 10^{5.5}$	$\geq 10^{5.3}$
I	16 hrs.	$10^{6.0}$	$10^{6.5}$
	24 hrs.	$10^{6.3}$	$10^{6.25}$
II	16 hrs.	$10^{6.67}$	$10^{6.0}$
	44 hrs.	$10^{6.0}$	$10^{5.25}$
Cell-free virus	1 hr.	$10^{6.0}$	

* All infected cultures and cell-free virus washed 2x 1 hr. after infection.

of each wash, the "pellet" from 1 tube was mixed with 1 ml BSS-5C and frozen. All were titrated in L cell tube cultures. The unwashed virus had a titer of $10^{8.2}$ ID_{50}/ml, while after 8 washings there was a residue of $10^{3.0}$ ID_{50}/ml.

In view of the above, two sublines of EAT cells adapted to <u>in vitro</u> growth were obtained from Dr. G. E. Foley (Children's Cancer Research Foundation; Boston, Mass.). These were arbitrarily designated by us as EAT-F and EA-2. In order to determine the suitability of these cells for our studies, crude and semipurified IF preparations were tested for their antiviral effects in monolayer cultures as compared with those in L cells. Results of challenge of the pretreated cells with Mengovirus are shown in Table 6. Repitition of this experiment with a different IF - containing CM Sephadex eluate gave comparable results. Since the semipurified IF was not protective of these cells, they obviously could not be utilized for study of an antagonist.

Retrospectively, a point of technic was considered in evaluating the results with these cells. The assay cultures were exposed to the IF preparations only 2 days after initiation and were challenged the following day. It is to be noted that Cantell and Paucker (3) reported the age of S3 clone of HeLa cell cultures to profoundly affect their sensitivity to IF, 1 to 2 day cultures requiring much larger amounts than did older cultures. With this in mind, varying dilutions of crude and semipurified IF were added to 2 groups of EAT-F monolayer cultures which respectively

Table 6. Assay of crude and semipurified IF in L, EAT-F and EA-2 monolayer cultures with Mengovirus challenge.

Cell line	IF	Crude IF Average % CPE, day: 2	3	5	6	7	Semipurified IF Average % CPE, day: 2	3	5	6	7
L	1:100	0	0	0	0	0	0	0	0	0	0
	1:200	0	0	0	<1	<1	0	0	0	0	<1
	1:400	0	0	0	<1	2	0	0	0	0	1
	None	75	100				75	100			
EAT-F	1:100	0	0	100			0	0	100		
	1:200	0	0	100			0	5	100		
	1:400	0	1	100			0	5	100		
	None	0	27	100			0	27	100		
EA-2	1:100	0	3	94	100		0	62	100		
	1:200	0	50	100			0	50	100		
	1:400	0	56	100			0	62	94	100	
	None	0	86	100			0	86	100		

had been initiated 2 and 6 days previously. After overnight incubation, cultures were challenged with $10^{2.0}$ ID_{50} of vesicular stomatitis virus (as titered in L cells). Cytopathic effects of this inoculum were minimal in control cultures. For this reason, cultures were frozen 4 days after challenge, and lysates of pooled replicate cultures were subsequently assayed in L cells for infectious virus. Results, shown in Table 7, confirm our former conclusion of the unsuitability of these cells for our purposes.

Another line of EAT cells, adapted to growth in suspension cultures, was obtained from Dr. C. A. Hirsch (Beth Israel Hospital; Boston, Mass.). Dr. Hirsch commented that these cells adhered poorly to solid substrates. We found this to be true when the cells were planted in plastic tissue culture flasks, but that adherent cell sheets formed in glass vessels.

Cultures of these cells - designated by us as EAT-S - were found highly susceptible to Mengovirus, which produced distinct cytopathic changes in cultures in fluid medium and discrete plaques under agar. In addition, semipurified interferon protected cultures against challenge with this virus.

These cells were then used in spinner cultures to look for IF antagonism in our CM Sephadex column eluates. Groups of 10 consecutive fractions, collected before IF elution, were pooled in equal volumes. One ml aliquots of semipurified IF were added to 1 ml of each respective pool. EAT-S cells plus SSM were added to

Table 7. Replication of vesicular stomatitis virus in EAT-F cells pretreated with crude and semipurified IF.

Experimental system			Yield of VSV**
Culture age*	Pretreatment		(ID_{50}/ml)
2 days	Crude IF	1:25	$10^{6.0}$
	Crude IF	1:50	$10^{6.5}$
	Purified IF	1:25	$10^{6.5}$
	Purified IF	1:50	$10^{6.5}$
	No IF		$10^{7.0}$
6 days	Crude IF	1:25	$10^{6.5}$
	Crude IF	1:50	$10^{6.5}$
	Purified IF	1:25	$10^{6.0}$
	Purified IF	1:50	$10^{6.5}$
	No IF		$10^{6.5}$

* Culture age at time of IF treatment

** Cultures frozen 4 days after challenge

the mixtures to give a total of 20 ml. The final cultures consisted of 1:20 dilutions of IF and test pools and of 2.85 x 10^5 cells/ml. Appropriate IF and cell-virus control cultures were also initiated. All mixtures were incubated with continuous stirring for 24 hours and cells sedimented by centrifugation. Each cell pellet was resuspended in 4 ml SSM and challenged with $10^{8.5}$ ID_{50} of Mengovirus. After 45 minutes, cells were washed 3 times, resuspended in SSM (20 ml/ culture) and reincubated 16 hours with stirring. Cultures were centrifuged, supernates discarded and washed cells resuspended in 4 ml SSM. The suspensions were subjected to 2 freeze-thaw cycles and aliquots were refrozen for subsequent assay in L cells for infectivity. Results are shown in Table 8.

It is seen that all eluate pools exhibited some inhibitory activity. This was most notable in pool A, but the effect was much less than that reported by Truden et al.

This experiment was repeated with pools of fractions obtained by chromatography of the crude IF induced in the presence of labelled amino acids. Unlabelled IF and EAT-S cells were employed. The extent of replicated virus is shown in Table 9. As with the unlabelled eluates, antagonist activity was negligible or absent, and not correlated with radioactivity.

These results were indicative of lack in our preparations of significant IF antagonist effective in EAT-S cells. Crude IF was therefore assayed in EAT-S monolayer bottle cultures. The cultures

Table 8. Assay of CM Sephadex eluates from unlabelled IF for antagonist to IF inhibition of Mengovirus replication in EAT-S cells.

	Pretreatment of EAT-S cells.	Virus yield (PFU/ml)
A.	IF + fractions 1-10	4.1×10^6
B.	IF + fractions 11-20	1.8×10^6
C.	IF + fractions 21-30	2.4×10^6
D.	IF + fractions 31-40	2.4×10^6
E.	IF + fractions 41-50	1.2×10^6
F.	IF + fractions 51-60	1.4×10^6
G.	IF + diluent	6.0×10^5
H.	Diluent	6.8×10^6

Table 9. Assay of CM Sephadex eluates from labelled IF for antagonist to IF inhibition of Mengovirus replication in EAT-S cells.

	Pretreatment of EAT-S cells	Average DPM/ml/fraction	Virus yield (PFU/ml)
A.	IF + fractions 1-10	7,041	18×10^6
B.	IF + fractions 11-20	10,622	4×10^6
C.	IF + fractions 21-30	763	5.9×10^6
D.	IF + fractions 31-40	ND	14.7×10^6
E.	IF + fractions 41-50	221	3.7×10^6
F.	IF + fractions 51-60	90	16×10^6
G.	IF + fractions 61-70	50	12×10^6
H.	IF + fractions 71-80	26	6×10^6
I.	IF + diluent		7.3×10^6
J.	Diluent		10.7×10^7

were pretreated with 4 ml amounts of varying dilutions of (a) interferon or (b) control medium harvested from L cells. Additional cultures were pretreated with diluent alone. After overnight incubation, test preparations were washed away and all cultures were challenged with Mengovirus expected to yield 50 - 100 PFU/control culture. One hour later the residual inoculum was removed and cultures were overlaid with nutrient medium containing 1% agar and protamine sulfate at a concentration of 400 ug/ml. Cultures were further incubated for 3 days, stained with neutral red and plaques counted. Results are shown in Table 10. The titer of the crude IF was 640 PDD_{50} units/4 ml.

The comparative susceptibility of EAT-S and L cells to the protective action of IF was then determined. Monolayer cultures were pretreated with varying dilutions of crude IF, challenged, overlaid, and stained as above. Resulting plaque counts are shown in Table 11. The titer of this crude IF was clearly the same in both host cell assay systems.

The possibility that mouse passage of the EAT-S cells might alter their susceptibility to crude IF and render them useful in the search for an IF antagonist was investigated. After 3 serial passages of the EAT-S cells in mice, cells from ascitic fluid were used to initiate monolayer cultures. These, in parallel with cultures of the non-mouse passaged EAT-S cells, were employed in the assay of crude IF as above. Results are presented in Table 12. The protective effect of the IF in mouse passaged cells

Table 10. The effect of pretreatment of EAT-S menolayer cultures with crude IF on subsequent plaque production by Mengovirus.

Cultures pretreated with:		Average number plaques/culture
Diluent		69
Control medium	1:20	57
Control medium	1:40	70
Control medium	1:80	66
IF	1:20	0
IF	1:40	0
IF	1:80	5
IF	1:160	7
IF	1:320	20
IF	1:640	25
IF	1:1280	38

Table 11. Titration of crude IF in monolayer cultures of EAT-S and L-929 cells.

Pretreatment of cultures	Average PFU/culture	
	L-929	EAT-S
IF 1:160	35	0
IF 1:320	35	16
IF 1:640	44	19
IF 1:1280	52	55
IF 1:2560	66	60
IF 1:5120	72	ND
Diluent	82	73

Table 12. Titration of crude IF in monolayer cultures derived from EAT-S cells passaged *in vitro* and *in vivo*.

Pretreatment of cultures.	Average PFU/culture, cells passaged.	
	in vitro	*in vivo*
IF 1:50	0	0
IF 1:100	0	0
IF 1:200	0	0
IF 1:400	0	0
IF 1:800	3	0
IF 1:1600	29	0
Diluent	112	153

was equal to or greater than that in the in vitro passaged cells as determined by the plaque reduction assay technic.

In a parallel experiment, the effects of crude IF pretreatment of mouse-passaged and of in vitro passaged EAT-S cells on the replication of Mengovirus were compared. The results of plaque assays of total (intracellular and extracellular) virus yields are given in Table 13. By this assay technic, the protective action of IF in cultures derived from the mouse-passaged cells was greater than that in the in vitro passaged cells, confirming the above results.

Employing a variety of cell systems and technics of assay, we have been unable to demonstrate the presence in NDV - induced IF produced in L cells of an IF antagonist active in the various Ehrlich ascites tumor cells available for our use. The reason(s) for this discrepancy with the results of previous work in this laboratory is (are) unknown. The NDV and Mengovirus which we employed were propagated (with minimal passage) from their stock, and their technics of IF induction and chromatographic separation were followed. Two deviants existed, however. (1) We obtained our L-929 cells from the American Type Culture Collection Cell Repository; theirs were obtained from another laboratory. (2) The mouse passage of their EAT cells had been discontinued before our studies began; our EAT cells came from a variety of sources.

In respect to these variants, it is well known that the characteristics of any cell line or subline reflect not only the

Table 13. The effect of pretreatment with crude IF on the subsequent replication of Mengovirus in monolayer cultures initiated from *in vitro* and *in vivo* passaged EAT-S cells.

Cultures from cells passaged:	Pretreatment of cultures	Virus yield, PFU/ml
In vitro	IF	2.8×10^4
In vitro	Diluent	2.7×10^6
In vivo	IF	$< 3 \times 10^1$
In vivo	Diluent	3.0×10^5

tissue of origin but also its entire cultural history. It is possible that serial subculture of the L cells used previously effected selection of a variant with unusual capacity to elaborate an intraspecies IF antagonist - or, conversely, our cultures consisted of variant cells which lacked such capacity. On the other hand, we have demonstrated that the various EAT (or EAT-derived) cells tested by us differed markedly in their susceptibility to the protective action of semipurified IF. The thesis that they differed from those of Truden et al. in their capacity to express the action of an IF antagonist therefore is not an untenable one.

II. Investigations of the effects of chick chorioallantoic membrane extracts on enterferon action.

During the course of the above studies, Fournier et al. (4) demonstrated the presence in extracts of human chorionic or amniotic membranes of a tissue antagonist of interferon activity which they designated TAI. We undertood and investigation of whether extracts of chick chorioallantoic membranes would have a similar effect.

Membranes harvested from embryonated eggs which had been incubated for 12 days were washed in balanced salt solution, lyophilized, then pulverized by grinding. The powder was rehydrated in Eagle's minimum essential medium (MEM) at a concentration of 2 gm of dry weight/100 ml and agitated with a magnetic stirrer for 18 hours at 4°C. The extract was clarified by low speed centrifugation then by high speed centrifugation (90,000 g, 4 hours) in a Spinco Model L centrifuge. The resulting preparation , designated CAME, was stored at 4°C.

To assay the activity of this preparation, 2 - fold dilutions of NDV - induced L cell IF were made. To 5.7 ml aliquots of each dilution was added 0.3 ml of CAME or of diluent. Growth medium was decanted from monolayer tube cultures of L cells and replaced with 1 ml of IF-CAME, IF-diluent or of diluent (MEM with 5% fetal calf serum: "M5C"). After overnight incubation, test preparations were replaced with M5C and cultures were challenged with 10^3 ID_{50} of Mengovirus. Two days later, when virus controls exhibited 4+ CPE,

cultures pretreated with the 1:250 dilution of IF, with and without CAME, and virus control cultures were frozen. Subsequently, replicates were pooled and assayed for infectious virus. Results of microscopic observations and of plaque assays are shown in Table 14. In the absence of IF the CAME had no antiviral effect, but it clearly potentiated IF activity.

The effects of trypsin, heat and dialysis on the CAME were determined. (a) To an aliquot of undiluted extract was added an equal volume of 2X crystallized trypsin (200 ug/ml) at alkaline pH. The mixture was incubated at 37°C for 1 hour and then heated at 56°C for 30 minutes. (b) Undiluted CAME was heated at 56°C for 30 minutes. (c) Undiluted CAME was dialyzed overnight at 4°C against 400 volumes of balanced salt solution. These preparations were mixed with IF and M5C to effect a 5% final CAME concentration and a 1:200 dilution of IF. One ml amounts were added to monolayer tube cultures of L cells. After overnight incubation, test mixtures were replaced with M5C and cultures were challenged with approximately 10^3 ID_{50} of Mengovirus. When virus controls showed 4+ CPE, cultures were frozen, Lysates from replicate cultures were subsequently pooled and assayed in L cells for infectious virus. Results, presented in Table 15, showed heat and trypsin to be without effect on the active moiety of the CAME, while dialysis diminished but did not abolish it.

An investigation of the temporal relationship between the CAME and IF actions indicated the effectiveness of CAME in potentiating

Table 14. The effect of CAME on the antiviral effects of crude IF in L-929 cultures.

	With 5MCS		With CAME	
II diln	Average % CPE day 2	PFU/ml harvest	Average % CPE day 2	PFU/ml harvest
1:250	< 1	6.5×10^5	Neg.	3.1×10^4
1:500	< 1		< 1	
1:1000	14		8	
1:2000	24		14	
1:4000	79		69	
No IF	100	4.9×10^8	100	4.5×10^8

Table 15. The effects of trypsin, heat and dialysis on the IF potentiating activity of CAME

Pretreatment of cultures	Virus yield, PFU/ml
M5C	1.9×10^8
M5C + untreated CAME	1.5×10^8
IF + M5C	8.1×10^4
IF + untreated CAME	5.9×10^3
IF + trypsin-treated CAME	4.5×10^3
IF + heated CAME	3.2×10^3
IF + dialyzed CAME	1.7×10^4

the antiviral effect of IF was demonstrable only when cultures were simultaneously exposed to the two entities. This was shown by the fact that virus replication in cultures consecutively treated with CAME then IF prior to infection was comparable to that in cultures treated with IF alone.

The eggs from which the CAME used in the above experiments was derived were from a local commercial hatchery and were from conventional flocks of chickens. Since many such flocks are carriers of viruses of the avian leukosis-sarcoma complex as well as that of Marek's disease, and since these viruses can be transmitted through ova, there existed the possibility that one or more latent viruses in the embryos could be responsible for the CAME action. Additional extracts were prepared and tested as above at various times. Some of these were derived from conventional eggs and some from Spafas eggs (from flocks free of agents of the avian leukosis-sarcoma complex).

A total of 10 such preparations have been tested. In the absence of exogenous interferon, 8 were without direct effect on Mengovirus replication, and the other 2 reduced virus growth only slightly. When virus yields from cultures pretreated with IF alone were compared with those from cultures pretreated with IF plus CAME, varying effects of the different CAME preparations on IF action were observed. (1) Two were without effect. (2) Three exhibited distinct IF antagonism. (3) In contrast, 5 markedly augmented the antiviral effect of IF. It is to be noted that of 5

preparations derived from Spafas eggs, 3 exhibited IF antagonism while 2 enhanced IF action.

These results argue against the divergent effects being due to contamination of some of the preparations with latent avian viruses, as does the fact that the extracts exhibited little or no effect in the absence of exogenous IF. It is possible that the CAME preparations contain multiple substances, present in varying proportions, which separately act antagonistically or in concert with IF.

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