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Enhancement of Phagocytosis by Interferon-Containing Preparations

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Exposure of mononuclear cells from the mouse peritoneal cavity to interferon (IF)-containing mouse sera enhanced phagocytosis of colloidal carbon particles by the cells. The same effect was observed when the cells were exposed to IF-containing cell culture harvest free of serum. The magnitude of this effect of IF-containing preparations paralleled the titer of IF and was not related to the dilution of various IF-containing serum specimens tested. The factor responsible for the enhancing effect was stable at *p*H 2, inactivated by trypsin, and nonsedimentable at 105,000 × *g*. Heating at 60 C for 1 hr destroyed it, and its kinetics of heat inactivation paralleled that of the antiviral activity of IF. A period of incubation of phagocytic cells with IF-containing serum was necessary before a maximum level of enhancement was reached, and once established was not removable by repeated washing of cells. The kinetics of the production of the enhancing factor in mice injected with Newcastle disease virus was essentially identical to that of the simultaneous production of IF as measured by antiviral activity. Contrary to the effect of mouse IF preparations, human IF preparation did not enhance the activity of mouse phagocytes. It appears, therefore, that the phagocytosis-enhancing factor falls within the present definition of IF.

Infections in experimental animals or in ovo, caused by several nonviral pathogenic agents, have been shown to be suppressed by prior injection of interferon (IF) or its inducers. They include bacterial agents such as *Escherichia coli* (20), *Listeria monocytogenes* (16), and *Klebsiella pneumoniae* (15), *Chlamydia* (12), and the protozoan parasite, *Plasmodium berghei* (9, 10, 17). Some of these and others have been similarly affected by IF in cell culture. An explanation of the suppressive effects of these pathogenic agents, entities with far more complex biochemical activities than viruses, is not provided within the current hypothesis of the action of IF, developed from the studies of its antiviral activity (11, 14, 18). Remington and Merigan (16) reported that macrophages from mice injected with pyran had, in comparison to uninjected controls, greater ability to take up *L. monocytogenes*, measured 1 hr after the bacteria were added to the cells. These authors discredited IF as the factor responsible for the "activation" of macrophages. On the other hand, circumstantial evidence is increasing to indicate an intimate relationship between IF and functions of lymphoid and reticuloendothelial elements (4–6).

It is well documented that fixed phagocytes play an important role in resistance of the vertebrate host to malaria parasites, and it was postulated by us previously (17) that the protective action of IF inducer in the malaria-infected mouse might be mediated through phagocytic cells. This report describes experiments in which IF-containing preparations were found to enhance the uptake of carbon particles by mouse mononuclear phagocytes in vitro.

MATERIALS AND METHODS

Mice. Adult, female NMRI Swiss mice weighing 20 to 22 g were used for IF production and to obtain peritoneal macrophages for in vitro studies.

Viruses. The strains, sources, methods of preparation, and titrations of stock Newcastle disease virus (NDV) and vesicular stomatitis virus (VSV) have been described elsewhere (8).

Interferons. Mouse IF, induced by NDV in L-cell cultures, and appropriate control culture medium were kindly supplied by T. C. Merigan of the Stanford University Medical School. It was reported that the IF-

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containing harvest had been held at pH 2 in the refrigerator for 5 days and centrifuged. It was pressure-dialyzed in a UM-10 ultrafiltration membrane (Amicon) which resulted in an eightfold concentration and a titer of 2,550 units/ml. The control preparation was from an unprocessed harvest from noninduced cultures.

Human IF, derived from human leukocyte cultures, with a reported titer of 15,000 units/ml, and a normal control consisting of unstimulated leukocyte culture fluid were kindly provided by J. Pagano of Smith Kline and French Laboratories.

Batches of stock mouse serum IF (MSIF) were prepared in this laboratory by injecting mice intravenously with 0.4 ml of the undiluted NDV-allantoic fluid and harvesting the blood for serum by decapitation 10 hr later. Mice inoculated with normal allantoic fluid were similarly processed to provide control serum (CS). These sera were either acid-treated for 5 days (batch 2) or centrifuged for 90 min at 105,000 × g (batches 15 and 17) to inactivate or eliminate the inducing virus and were stored at −70 C until used. Assays of these stock MSIF and specimens which required IF determinations were performed by the plaque reduction method previously described (8) or by extinction of inhibition of cytopathic effect in L-cell tube cultures with VSV as the challenge virus. When the latter method was used, the titer of IF was defined as the reciprocal of the highest dilution of IF which inhibited 50% of the cytopathic effect.

Cultivation of macrophages and assay of phagocytic activity. Phagocytic cells were collected from mouse peritoneal cavities by techniques similar to those described by Tegtmeyer and Craighead (19). Two milliliters of culture medium that consisted of Eagle's minimum essential medium (MEM) with 10% horse serum and antibiotics was injected intraperitoneally into each mouse. After 1 min, the animal was killed and a laparotomy was performed. The peritoneal fluids were removed and collected in a pool. Fibrin clots were removed from the pools of fluid, the cells were well mixed, and 1-ml quantities were placed into flat-bottom tubes (diameter = 13 mm) with cover slips. The tubes were centrifuged lightly (400 rev/min, 5 min) to sediment the cells and promote adherence. After incubation for 2 hr at 37 C, the cells were washed three times and further incubated in the medium for 3 to 4 days. Culture fluids were then replaced with selected dilutions of IF-containing samples or a corresponding control, two or more tubes per dilution. In each experiment, the control preparation was diluted to the same extent as the test preparation. After 24 hr of further incubation, the medium was again replaced with fresh medium containing a 1:1,200 dilution of colloidal carbon (Pelikan, Gunther Wagner). After incubation at 37 C in a water-bath shaker (33 rev/min) for the desired time intervals, the cells were washed, fixed in 100% methanol, stained by Giemsa, and examined microscopically (× 970). All specimens were coded by one individual and read "blind" by a second. Occasional tubes in which cells had not adhered satisfactorily or were obviously unhealthy were discarded. Cells containing clearly recognizable aggregates of carbon were considered to be actively phagocytizing (Fig. 1). The phagocytic activity of a particular specimen was expressed as the percentage of phagocytizing cells in 500 to 1,000 total cells observed that were distributed in 40–80 microscopic fields.

It should be noted that the cells, in all experiments, were exposed to 10% horse serum in the MEM, and

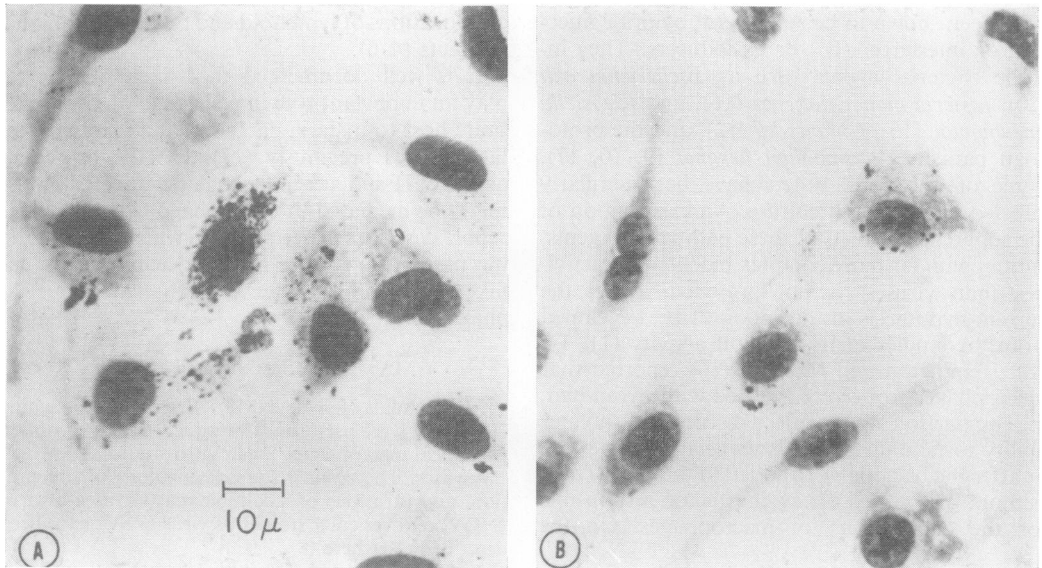

FIG. 1. *Cells from mouse peritoneal cavity with phagocytized carbon particles. The cells were incubated in vitro with IF-containing mouse serum (A), or CS (B) before addition of carbon. Wright-Giemsa stain. All cells in A and 2 cells in B would have been scored as positive for phagocytosis.*

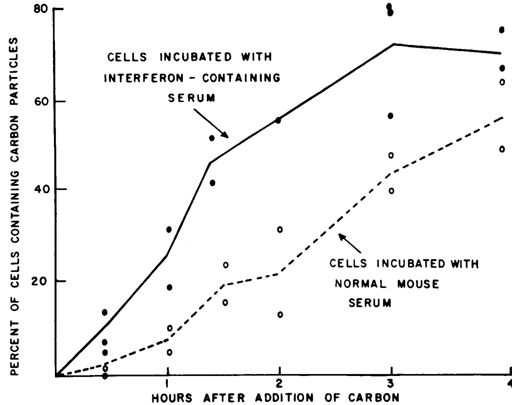

FIG. 2. *Effect of IF-containing serum on ingestion of carbon particles by mouse phagocytic cells in vitro. Dilutions of sera (1:833, to provide 60 units of IF) were added to incubating cells 24 hr before addition of carbon.*

that the dilutions of mouse serum preparations to provide, e.g., 50 units of IF were as great as 1:900. Therefore, the composition of medium to which the cells were exposed was quite uniform and the exposure of cells to significantly different concentrations of total protein was avoided.

RESULTS

An experiment (experiment 1) was carried out in which duplicate or triplicate specimens were fixed at several intervals of time after addition of carbon, in an attempt to determine the optimal time of evaluation of phagocytic activity. Sixty units of batch 2 MSIF was used as the test material, and the control tubes received a similar dilution (1:833) of acid-treated CS. As depicted in Fig. 2, the rate of uptake of carbon by MSIF-treated cells was significantly higher than by control cells: a plateau was reached at 3 hr in the former, whereas in the latter the number of cells showing activity increased at a constant but slower rate. Since the difference in the phagocytic activity of the two groups was most pronounced at approximately 2 hr after the addition of carbon, subsequent observations were made only on specimens fixed after 2 or 2.5 hr of incubation.

Figure 3 shows the results of a dose-response study. Phagocytes were incubated overnight with 1, 6, 12, 25, 50, 100, or 200 units of MSIF no. 2. For controls, CS diluted to the equivalent of 1 and 200 units of IF was used. The phagocytic activity increased in response to increasing concentrations of IF until a maximum was reached with the concentration of IF at approximately 25 units/ml. The presence of excess IF did not elicit substantial further increase in phagocytosis. Therefore, in subsequent experiments MSIF at concentrations of 25 to 50 units/ml, depending primarily on the convenience of diluting a particular batch of IF, was used to obtain maximum phagocytosis.

Table 1 summarizes results obtained in experiments designed to confirm those of experiment 1 and to test MSIF prepared by different procedures from different sources. Results similar to those seen in experiment 1 were again obtained with MSIF no. 2 (experiment 4) and with two other preparations of mouse IF. Batch 15, NDV-induced MSIF and its control, which were subjected only to centrifugation at 105,000 × *g* for 90 min to remove any residual virus, gave results in experiments 2, 3, and 5A which confirmed those of experiment 1. The IF-containing cell culture harvest also enhanced phagocytosis (experiment 6). The differences between IF-containing and control preparations are emphasized by the observation that cultures containing control material showed a level of phagocytosis similar to that of the tubes that received MEM only. This is apparent in experiments 2 and 5A and somewhat less so in experiment 6. A 25-fold greater dilution reduced the phagocytosis-enhancing effect of the MSIF in experiment 3, but did not change the level of phagocytosis seen with CS. A similar but less clear-cut effect of dilution was seen in experiment 4.

Because results from these preliminary experiments suggested a definite relation between the phagocytosis-enhancing activity and IF, the enhancing factor was subjected to the following characterizations.

Sensitivity to heat. Mouse IF preparation no. 15 was subjected to heating at 60 C for 1 hr and

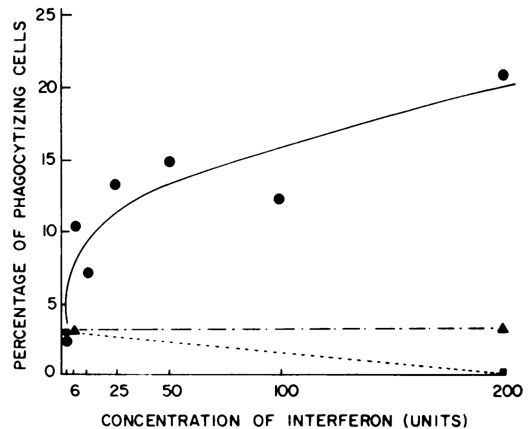

FIG. 3. *Dose-response curve of the phagocytosis-enhancing effects. Phagocytes were incubated with indicated units of MSIF or CS for 24 hr before carbon was added. Circles, MSIF; squares, CS; triangles, MEM.*

TABLE 1. *Effects of interferon (IF)-containing preparations on phagocytic activity of mouse phagocytic cells*

Expt	Test prepn		Percentage of cells with phagocytized carbon after exposure to		
	Source	IF titer (U/ml)	IF-containing preparation	Control preparation	Minimal essential medium only
2	15, Mouse serum IF	50	37[a], 50	10, 22	19, 17
3	15, Mouse serum IF	50	64, 54	21, 20	
	15, Mouse serum IF	2	33, 37	19, 25	
4	2, Mouse serum IF	50	20, 23	11, 9	
	2, Mouse serum IF	2	8, 8	4, 5	
5A[b]	15 Mouse serum IF, unheated	50	30, 20, 16	5, 9, 1	3, 3
5B	5, Mouse serum IF, heated	50	3, 1, 6	1	
5C	15, Mouse serum IF, cells washed	50	12, 27, 10		
5C	15, Mouse serum IF, cells washed	2	1, 5		
6	Cell culture IF	50	51, 50	20, 23	16, 10

[a] Each figure corresponds to one tube.

[b] Experiment 5, code A served as control for comparison with sera that had been heated at 60 C for 1 hr (code B) and for code C in which the cells were washed three times after exposure to the test preparation.

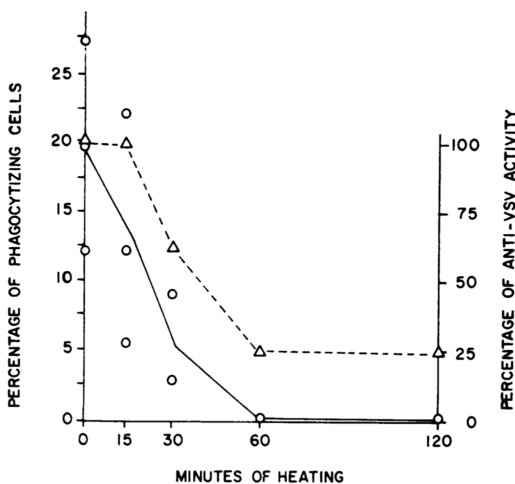

FIG. 4. *Kinetics of heat inactivation of the phagocytosis-enhancing factor and antiviral activity of interferon. Mouse serum IF was heated at 60 C and, at indicated intervals, samples were assayed for phagocytosis-enhancing (circles) and antiviral activity (triangles).*

tested (experiment 5B) in parallel with unheated preparation (experiment 5A). The result (Table 1) shows that the phagocytosis-enhancing factor was completely destroyed by the heating. Further examination of the effect of heat is presented in Fig. 4. Mouse serum IF no. 17 was heated at 60 C and, at intervals of 0, 15, 30, 60, and 120 min, samples were taken and tested for antiviral and phagocytosis-enhancing activities. The results show (Fig. 4) that both activities declined in a parallel fashion. The enhancing factor again was removed completely by 60 min at 60 C.

Effect of washing. Phagocytes incubated with MSIF no. 15 were washed three times with medium before addition of carbon. As shown in Table 1, experiment 5C, washing failed to remove the enhancing effect of MSIF. The enhancing activity therefore becomes closely associated with the cell.

Requirements for preincubation. To determine whether a period of preincubation is required for the phagocytosis-enhancing effect to reach its maximum, triplicate specimens were incubated for 0, 1/3, 2, 4, 12, 18, and 24 hr with MSIF no. 17 before carbon was added. As illustrated in Fig. 5, enhanced activity was not demonstrable if the incubation period was 4 hr or shorter. The enhancement became apparent between 4 and 12 hr of incubation and rapidly increased thereafter.

Sensitivity to trypsin. A portion of MSIF batch no. 17 was diluted to a concentration equivalent to 500 units/ml in MEM without serum. This relatively high concentration was used so that samples could be further diluted to obtain uniform composition in all test materials to be added to phagocytes.

Stock solutions of 2× crystalline trypsin and 5× crystalline soy bean trypsin inhibitor (SBTI, Nutritional Biochemical Corp.) were prepared in MEM immediately before use in concentrations of 10 mg/ml and 40 mg/ml, respectively. A 2-ml amount of the diluted MSIF was incubated at 37 C for 2 hr with 0.04 ml of trypsin solution. At the end of the incubation, 0.015 ml of SBTI was

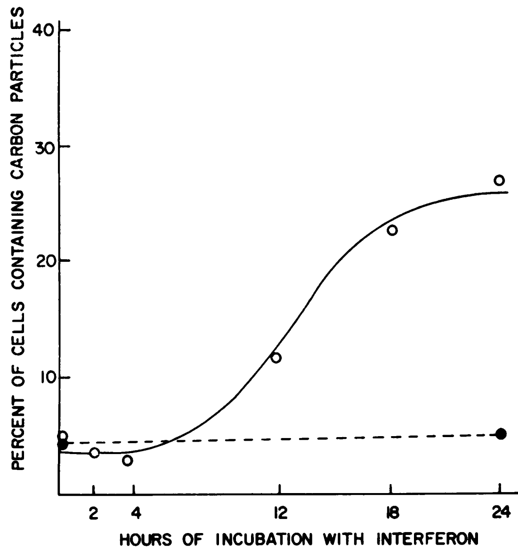

FIG. 5. *Effect of the length of time of incubation on the phagocytosis-enhancing effect of MSIF. Phagocytes were incubated with MSIF for the indicated duration of time before carbon was added. Open circles, MSIF; closed circles, CS.*

added, and the reaction mixture was further incubated for 15 min after which a dilution of 1:10 in MEM was made for assay of the enhancing factor. A similar procedure was carried out in each of the control mixtures (Table 2). Where one or two components of the reaction mixture were deleted, MEM without serum was substituted. The overall activity obtained in this experiment was lower than those obtained in other experiments, but it is clear (Table 2) that the phagocytosis-enhancing activity of the serum was significantly reduced by trypsin.

Kinetics of the in vivo production of phagocytosis-enhancing factor and IF. An attempt was made to compare the kinetics of the production in mice of enhancing factor and of IF. Mice were injected intravenously with NDV, and at intervals groups of 8 to 10 mice were sacrificed, blood was collected, and serum was separated. Control specimens were prepared from mice injected with normal allantoic fluid. Each serum specimen was centrifuged at 50,000 × *g* for 90 min, serially diluted, and titered for IF and for phagocytosis-enhancing activities. To compute the titer of the enhancing factor, the degree of phagocytosis in triplicate tubes was determined in the usual way. The incidence of cells that had phagocytized carbon particles in the tubes that contained control material was about 12%. Therefore, tubes in which 20% or more cells were phagocytic were scored as positive and those with fewer cells phagocytizing, as negative. The titer of a specimen was then expressed as the estimated dilution, determined by interpolation, giving an ED_{50}. The titers of both activities thus obtained are pre-

TABLE 2. *Effect of trypsin digestion on phagocytosis-enhancing effect of mouse serum interferon (MSIF)*

Reaction mixture	Percentage of cells with phagocytized carbon	Mean
MSIF[a], trypsin, SBTI[b]	1, 0, 3	1.3
MSIF, trypsin	3, 2, 0	1.6
MSIF, SBTI	23, 10, 2	11.6
Trypsin, SBTI	1, 0, 4	1.6
Trypsin	8, 1, 1	3.3
MSIF	20, 14, 8	14.0
NMS[c]	0, 0, 0	0
MEM	0, 0, 0	0

[a] MSIF diluted to 1:40 in minimal essential medium (MEM) without serum (500 units/ml) was used for digestion.

[b] Soy bean trypsin inhibitor (SBTI) was in all cases added at the end of an initial 2-hr incubation period.

[c] Normal mouse serum diluted to 1:40 in MEM without serum.

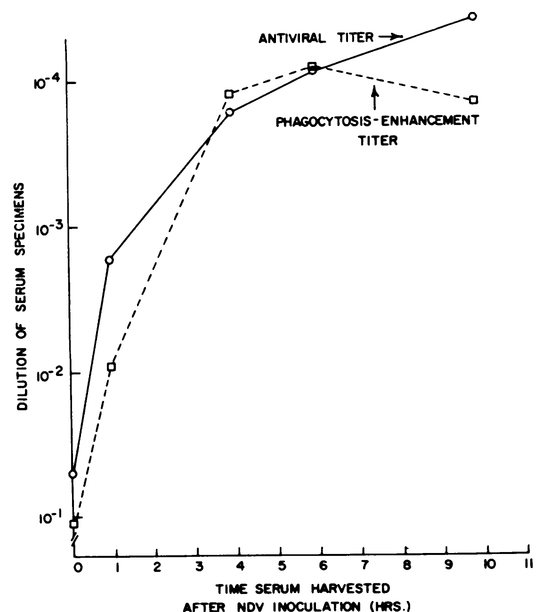

FIG. 6. *Kinetics of in vivo production of phagocytosis-enhancing factor, and of IF. Points indicate the 50% end point of each activity.*

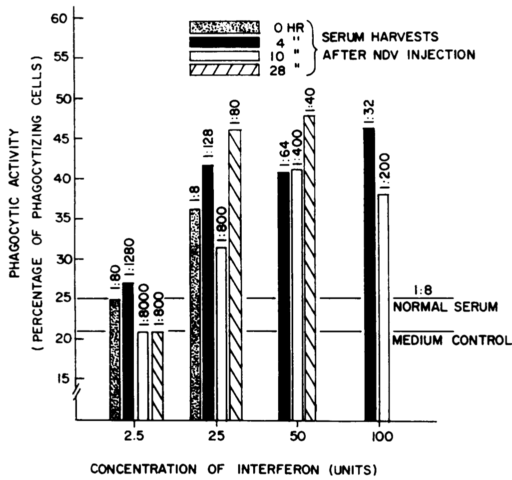

FIG. 7. *Relationship between concentration of IF and the phagocytosis-enhancing effect and the lack of relationship between the latter and the concentration of serum. The actual percentage figures of phagocytosis obtained in the experiment depicted in Fig. 6 are presented here in groups according to the concentrations of interferon that were used to treat the phagocytes. The dilutions of sera that provided the particular concentrations of IF are above the bars.*

sented in Fig. 6. The antiviral activity appeared rapidly upon stimulation with NDV, and by 8 to 10 hr it reached the peak. This pattern of response has been reported repeatedly and is well established. The induction of phagocytosis-enhancing factor followed a very similar curve. The low activities demonstrable in the initial specimens are not explained but are possibly related to virus still present in the circulation when the blood was collected and that was not removed by centrifugation at 50,000 × *g*. The results of these tests were then augmented by assays for phagocytosis-enhancing factor by using additional dilutions of selected serum specimens to provide the IF units (by antiviral assay) indicated in Fig. 7. In this series, 21 to 25% phagocytosis was observed in the control tubes. With these results presented in terms of units of IF present in the diluted specimen, it becomes more apparent that enhancing activity is related to the concentration of IF but not to the concentration of serum. For example, in Fig. 7 the dilutions of the serum specimens, collected at 0, 4, 10, and 28 hr, to obtain 2.5 units of IF were respectively 1:80, 1:1,280, 1:8,000, and 1:800, a difference by a factor of 100 between 0- and 10-hr specimens. Despite this magnitude of difference in the dilution, the phagocytic activities demonstrated by cells incubated with these specimens were quite similar, i.e., 24, 26, 21, and 21%. It may be noted that when median values of phagocytic activity of each IF dosage group in Fig. 7 are plotted and combined, a curve identical to that in Fig. 3 can be obtained.

Cell specificity. Host cell specificity, as determined by species or order, has long been held as one of the most important properties of IF, with some exceptions reported recently (1). Attempts to set up a system of phagocytic cells and IF of other animal species to study cell specificity of the enhancing factor have not met with sufficient success to permit the ideal "mirror" type experiments to be performed. Therefore, a human IF preparation and its control were used only with mouse macrophages to test for enhancing effect. The representative results of two experiments are presented in Table 3. The enhancing effect of MSIF is again clearly demonstrated in mouse cells. On the contrary, although both IF and control preparations from human cells in general enhanced the activity of mouse phagocytes, human IF in neither experiment exerted greater effect than the control preparation, nor did dilution of human IF preparation alter the enhancing effect. It was, therefore, concluded that human IF did not enhance the activity of mouse phagocytes.

DISCUSSION

It is apparent from these results that four different stock MSIF and several other IF-con-

TABLE 3. *Effect of human interferon (IF) preparation on mouse phagocytes*

Test preparations	Dilution	IF titer (units/ml)	Percentage of cells with phagocytized carbon
Human IF	1:300	50	30, 31, 29
Human IF	1:7,500	2	44, 21, 20
Human IF control	1:300		30, 44
Human IF control	1:7,500		41, 23, 16
Mouse serum IF	1:400	50	52, 42
Mouse serum IF	1:8,000	2.5	10, 17, 15
Normal mouse serum	1:400		19, 15
Normal mouse serum	1:8,000		10, 22, 24
MEM			11, 11, 18

taining experimental serum specimens, as well as cell culture harvest, were capable of enhancing the uptake of carbon particles by mouse phagocytes in vitro. The percentage of cells phagocytizing carbon was chosen as a method of objective quantitation, and no attempt was made in these experiments to estimate degrees of phagocytic activity in individual cells. Nevertheless, observers had the distinct impression that the amount of phagocytosis in individual cells was definitely greater in IF-treated cultures (Fig. 1).

When the various preparations containing the phagocytosis-enhancing factor (and IF) were subjected to the usual procedures associated with characterization of IF, the results gave no clue as to any property by which the two factors could be distinguished. These included stability at *p*H 2, nonsedimentability at 105,000 × *g*, inactivation at 60 C and by trypsin, a required incubation period after contact between cells and factor, and failure of removal from cells by washing. In addition, the kinetics of production of the two factors in mice were shown to be entirely parallel in an experiment which also clearly showed that the degree of phagocytosis enhancement was correlated with the titer of IF and not with the concentration of mouse serum. Although complete evidence concerning host cell specificity is not yet available, no enhancement of activity of mouse phagocytes with human IF of high titer was detected. The above observations, along with the facts that a consistent difference in phagocytosis enhancement was regularly seen between mouse IF-containing serum and CS, and that the factor was demonstrated in an IF-containing serum-free harvest from cell culture, are strong evidence against some factor other than IF in serum being responsible for the observations. Nevertheless, it may be helpful to point out that the effect is not attributable to two previously described phagocytosis-enhancing factors found in serum. Classical "opsonin" cannot be responsible because phagocytosis was evaluated by the uptake of inert carbon particles. Fidalgo and Najjar (2) isolated from dog and human gamma globulin a substance capable of enhancing the activity of homologous phagocytes. Tuftsin, as this substance has been designated recently, is a tripeptide, is present in normal serum, and is resistant to trypsin (13), characteristics that clearly differentiate it from the factor described here. These data taken together strongly favor the conclusion that the phagocytosis-enhancing factor described here is actually IF, utilizing the presently available descriptive definition. It is possible that the stimulus that results in IF production may initiate the formation of other factor(s) of similar chemical nature, responsible for the present observation. The answer to this question will depend upon studies with completely purified IF, not yet available. However, it should be emphasized that the enhancing factor is apparently completely dissociable from serum proteins, since an IF-containing serum-free preparation from cell culture was fully as active as serum IF preparations.

We postulated previously (17) that enhancement of phagocytosis may be the underlying mechanism by which IF renders mice more resistant to experimental mouse malaria. The data reported here support our speculation, although the previous observation suggested an effect on the parasitized erythrocyte rather than a direct effect on the phagocytes. In studying the effect of IF on mouse leukemia, Gresser et al. (6) found that ascitic fluid from mice treated with IF and then inoculated intraperitoneally with RC_{19} leukemic cells contained more macrophages with more active phagocytosis of the injected cells than did ascitic fluid from untreated controls. Their result is compatible with ours, although under their in vivo experimental conditions immunological and other undefined factors which were nonexistent in our system might have played important roles. The report of Remington and Merigan (16), in which no enhancement of uptake of *L. monocytogenes* by mouse macrophages treated with IF was observed, appears to contradict our result. However, detailed information on experimental conditions is unavailable for analysis. Also, Kazar et al. (10*a*) found no increased uptake of *Chlamydia trachomatis* in IF-treated cell monolayers and macrophages.

More recently, Gresser et al. (7) demonstrated that mouse IF preparations inhibited the propagation of mouse leukemia L 1210 cells and hypothesized that IF may have biological activities in addition to viral inhibition. Their report and our present results have strengthened our view (17) that IF induction, as determined by antiviral effect, may be only part of a basic process that provides the host with diverse protective effects against various invaders. A similar thought has also been expressed recently by Gifford et al. (3).

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