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Interferon Preparations Enhance Phagocytosis In Vivo

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Previous studies have shown that interferon (IF) preparations enhance phagocytic activity in cultured mouse peritoneal macrophages. It is shown here that cell culture fluids containing large amounts of IF, which had been treated with acid and clarified of the inducer, Newcastle disease virus, enhanced phagocytic activity when injected into mice. Enhanced phagocytic activity also was observed after injection of Newcastle disease virus into mice, but the contribution of IF to this event was unclear. The kinetics of the phagocytic response to inducers in vivo were biphasic. Depression of phagocytosis occurred around 16 to 18 h after injection of Newcastle disease virus. The observed enhancement began about 12 h later and lasted for at least 60 h more. It was concluded that the complexity of the response of mice to an inducer makes analysis of the role of IF in the ensuing events difficult. However, because of documented phagocytosis-enhancing effects of IF in vitro, it is very likely that the in vivo effects observed here are to some degree mediated by IF. On this basis, the concept of the activity of IF as a lymphokine is potentially expanded.

The role of interferon (IF) has been broadened recently by the association of its production with the stimulation of an immune reaction (27). This is most likely in connection with the phenomenon of blastogenesis (30; for review, see reference 2). In this regard, IF is released by the lymphocyte along with a number of other similar molecules collectively known as lymphokines (8). Functionally, IF does not relate to these lymphokines as a group. They are generally regarded as effector molecules in the events that lead to the establishment of cell-mediated immunity, whereas IF has been thought of as an agent of nonspecific immunity via its antiviral activity. A recent exception to this rule has been the description of type II IF wherein Salvin et al. (25) have demonstrated an association of IF with macrophage migration inhibitory factor (MIF).

Our recent evidence (9, 14) indicates that IF and a factor in IF preparations that enhances phagocytosis of carbon particles by mouse peritoneal macrophages in vitro are the same. The notion of IF as a lymphokine that alters macrophage function surely expands its potential as a mediator of immunological events.

However, from most evidence reported so far (4, 13, 24) it has been concluded that, after induction of IF in vivo, any enhanced reticuloendothelial activity is merely coincidental and that such activity is likely due to a prolonged, nonspecific stimulation of the reticuloendothelial system by the IF inducers themselves. This study was initiated to see whether IF, either induced or administered passively, can enhance phagocytic activity in vivo.

(This report constitutes part of a dissertation submitted to the Graduate School of Arts and Sciences, The George Washington University, in partial fulfillment of the requirements for the Doctor of Philosophy degree.)

MATERIALS AND METHODS

Viruses. An attenuated B-1 strain of Newcastle disease virus (NDV) was obtained from S. Baron, National Institutes of Health, Bethesda, Md. It was propagated in the allantoic cavity of embryonated hens' eggs that were 9 days old. Its infectivity titer was determined in chicken embryos to be $10^{8.3}$ 50% egg infectious doses per ml. Normal allantoic fluid (NAF) was used as a control preparation for this virus. The Indiana strain of vesicular stomatitis virus was supplied by G. Spahn, Microbiological Associates, Walkersville, Md., and was propagated in chicken embryo fibroblasts. It was shown to have an infectivity titer in L cells of 4.3×10^5 plaque-forming units/ml.

Mice. Female Swiss mice, NMRI strain, were obtained from the Naval Medical Research Institute, Bethesda, Md.

Cell cultures and media. The CCL-1 strain of clone-929 of the mouse fibroblast cell line (L cells) was obtained from the American Type Culture Collection, Rockville, Md. The C-243 cell line for high-yield production of IF (19) was obtained from S. Baron through G. Spahn.

Cultures were routinely grown on Eagle minimum essential medium (EMEM) supplemented with

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penicillin-streptomycin, L-glutamine, and fetal bovine serum, 10% by volume (EMEM-10), or were maintained on a similar medium with 2% serum (EMEM-2).

A special medium, conditioned medium, was used to culture macrophages. It contained EMEM, antibiotics, L-glutamine, 15% horse serum, and 20% of medium from confluent cultures of L cells that had been centrifuged for 20 min at 2,400 × *g* and passed through a filter with a pore size of 0.45 μm. The reagents above were obtained from Microbiological Associates, Bethesda, Md.

Media were also prepared to overlay cells with agarose. The reagents were from Grand Island Biological Co., Grand Island, N.Y. Medium 199 (2×), supplemented with 2% antibiotics and 20% fetal bovine serum, was mixed in equal parts with 2% melted agarose for the first overlay medium. A second overlay medium was the same, except 4% fetal bovine serum and 4% of a neutral red dye solution (3.333 g/liter) had been added to the medium before mixing with melted agarose.

Preparation of IF and mock IF. The C-243 cultures were grown to 90% confluency in 32-ounce (ca. 0.946 liter) glass bottles by using EMEM-10 as the culture medium. The cultures were washed with EMEM and 5 ml of NDV was added. After 1 h of incubation at 37 C, the virus was decanted and 20 ml of EMEM-2 was added. The culture fluids containing IF were collected 18 h after the addition of virus.

The IF was routinely clarified of cell debris by centrifuging at 2,400 × *g* for 20 min, then treated with 3 N HCl to effect a pH of 2.0, and held for 5 days at 4 C, after which the pH was adjusted to 7.0 with 3 N NaOH.

Sometimes the IF was concentrated by pressure dialysis in a filtration system (Amicon Corp., Lexington, Mass.) by using a UM-10 filter. A 10-fold concentration of the antiviral activity was effected by this process.

Mock IF was prepared by using the same procedures, except NAF was used as an inducer instead of NDV.

Antiviral assay for IF. A plaque reduction test for IF was developed using 24-well disposable plastic tissue culture trays and their tape sealers (Linbro Chemical Co., New Haven, Conn.; 1.5-cm diameter wells). Each well was seeded with 2.4×10^5 L cells in 1 ml of EMEM-10. Plates were sealed after planting and between test procedures, eliminating the necessity of an environment enriched with CO_2. One day after initiation of the cultures, samples of IF were serially diluted in EMEM-2, and each dilution was added to triplicate wells of washed L cells in 1-ml volumes. After 24 h of incubation at 37 C, each well of L cells was washed twice with EMEM and one-half of the control wells were inoculated with 0.2 ml of EMEM-2, while the remainder of the controls and all other wells were inoculated with 0.2 ml of vesicular stomatitis virus that had been diluted in EMEM-2 to contain 25 plaque-forming units/ml. The plates were incubated for 75 min at 37 C. Each well was washed three times with EMEM and supplied with 0.8 ml of the first overlay medium. The plates were incubated at 37 C for 24 h. Then, 0.4 ml of the second overlay medium was added to each well. Both overlay media were maintained as a liquid at 45 C during these procedures. After at least another 24 h of incubation at 37 C, the plaques were counted by using an opaque light source to illuminate the plates.

IF titers were calculated by the method of Reed and Muench for estimating 50% end points (23), wherein IF inhibited 50% of the plaques relative to the number seen in the control cultures infected with vesicular stomatitis virus.

In vivo phagocytic assay. A modification of the methods used in the previously described in vitro assay for phagocytic activity (9, 14) was employed for the collection of macrophages (adherent cells) for the in vivo assay. Carbon was diluted 1:2,000 in EMEM-2, and 0.2 ml was injected intraperitoneally (i.p.) into the mice. (The 1:2,000 dilution was determined to be the optimal amount for this assay in preliminary studies.) Thirty minutes later 2.5 ml of conditioned medium, which promotes rapid attachment of macrophages to glass, was injected into the peritoneum of each mouse. The mouse was killed, a laparotomy was performed, and the peritoneal fluids were removed with a capillary pipette. The fluid from each mouse was dispensed into glass tubes (1.5 by 5.5 cm) that contained a glass cover slip. These tubes were centrifuged at 120 rpm at 15 C for 5 min. They were cautiously washed to remove nonadherent cells, fed with the conditioned medium, gassed with a mixture of 5% CO_2 and 95% air, tightly stoppered, and incubated at 37 C for 2 h. (Some of the cultures at this point were exposed to a 1:1,200 dilution of the carbon for 30 min without agitation and then washed, stained, and examined for phagocytizing cells. Better than 98% of the cells proved to be phagocytic by this test, thus assuring that the phagocytosis assay was dealing primarily with macrophages.) They were then washed vigorously three times with EMEM, fixed with 100% methanol, and stained with Giemsa stain (Fisher Scientific Co., Pittsburgh, Pa.). The cover slips were thoroughly dried and mounted with Permount (Fisher) onto microscope slides. Each coverslip was coded and read "blind." The macrophages were examined microscopically under oil immersion, and phagocytic activity was determined as a percentage of the number of macrophages that contained carbon particles in relation to the total number of phagocytes counted. The phagocytic activity in the text is an expression of the mean phagocytic activity of all cultures in a test group. An expression of the relative phagocytic activity of a test group in relation to the control group was determined by the following formula:

$$\left(\frac{\text{Phagocytic activity in the test group [\%]}}{\text{Phagocytic activity in the control group [\%]}} - 1 \right) \times 100$$

= Relative phagocytic activity [%]

RESULTS

Kinetics of the phagocytic response in mice after intravenous injection of NDV. In five

separate experiments, 0.4 ml of NDV or NAF was injected intravenously into groups of three to five mice each. At intervals thereafter, the phagocytic activity of each mouse was determined. Representative data from experiment 5 are shown in Fig. 1. The kinetics of the phagocytic activity of mice that received virus was biphasic relative to those that received NAF. There was a period of depressed phagocytic activity 18 h after the injection. By 30 h, depressed activity changed to enhanced activity that lasted at least 66 h after the injection of NDV.

Kinetics of the phagocytic response in mice after intravenous injections of NDV 24 h apart. Experiments were conducted the same as those above, except mice received dual injections of various combinations of either NDV or NAF 24 h apart. It was hoped to determine whether the hyporeactivity phenomenon (the ability of an animal to respond with IF production to a proximate second induction stimulus) was functional, as regards phagocytosis-enhancing activity, in a manner similar to that seen in the classical antiviral IF system.

All mice received 0.4 ml of the inoculum intravenously, and the following groups resulted: first, mice received NDV and then 24 h later a second injection of NDV (group NN); first NDV and then NAF (group NA); first NAF and then NDV (group AN); first NAF and then NAF (group AA).

Two criteria were used to determine whether hyporeactivity was established. First, an antiviral assay was performed on the peritoneal fluids taken from the mice during the harvest procedure for establishing macrophage cultures. Second, an analysis of relative inflammation by polymorphonucleocytes (PMN) was made by examining blood smears prepared from a drop of tail blood taken shortly before the mice were killed to determine whether hyporeactivity of inflammation, as earlier described by Borecký and Lackovič (5), was evident. The results in Fig. 2 show that by both measures hyporeactivity was established. Mice that received a second injection of NDV (group NN) failed to respond with increased IF production or PMN inflammation.

The kinetics of the phagocytic response under circumstances of IF hyporeactivity were biphasic (Fig. 3). The magnitude of the response from 0 to 30 h was related to the regimen that each mouse had received. Mice in the AN group had the lowest initial response. Their phagocytic activity was depressed 16 h after the second injection relative to that of mice from group AA. The responses of the mice from the NN group and NA group were correspondingly

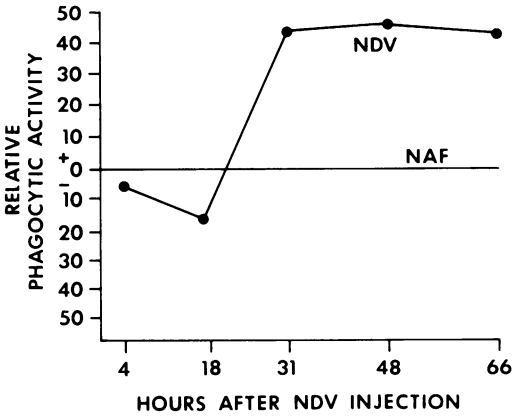

FIG. 1. *Kinetics of the phagocytic response in vivo: comparison of the effects of injection of NDV versus NAF into mice. Mice received injections of NDV or NAF, and their phagocytic activity was determined at the indicated intervals.*

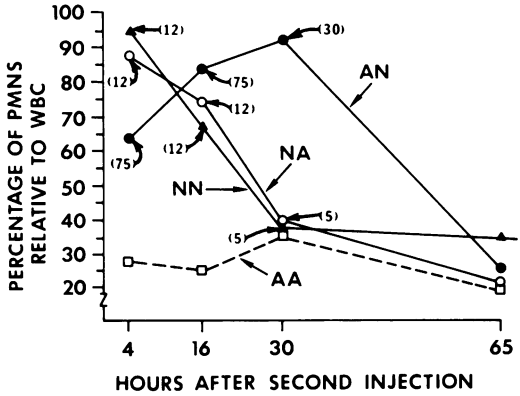

FIG. 2. *Kinetics of the inflammatory response: a comparison of the effects on mice of injections, given 24 h apart, of combinations of NDV and NAF, NDV and NDV, or NAF and NDV with mice injected with the combination NAF and NAF. Groups of mice received injections of NDV (N) or NAF (A), and 24 h later they received either the same or the reciprocal inoculum. The resulting groups were designated NA, NN, AN, and AA. Blood smears were taken at the indicated times after the second injections. These were stained and examined under oil immersion, and the percentage of PMNs relative to the total leukocyte (WBC) count was calculated. Figures in parentheses are the units of IF found in the peritoneal fluids of mice (per milliliter) at the indicated times in the indicated groups.*

higher during this time period. Relative depression of phagocytic activity at 18 h in both of these groups was still apparent, but it was not below the level of that seen in the control group, AA. After extended periods, mice in all groups that received NDV maintained an enhanced level of phagocytosis in relation to those

in group AA. In the mice in the NA group, enhanced phagocytosis was still apparent 89 h after the primary injection of NDV.

Effect of injection of IF preparations on phagocytic activity in vivo. Although phagocytosis was clearly enhanced following the induction of IF by virus, it was unclear whether this enhanced activity was in any way attributable to the action of IF. Therefore, studies on the effect of injecting IF preparations into mice on phagocytic activity were conducted.

Nine mice received injections of IF (10,000 U/mouse, 0.5 ml i.p.) or mock IF. Phagocytic activity was determined 12, 24, and 36 h after these injections. The results in Table 1 reveal that phagocytic activity was relatively low 12 h after these injections, especially in those mice that had received IF. At 24 and 36 h, there was little or no difference in the phagocytic activity in mice of either group. Concurrently, in the cultures established from both groups of mice 12 h after the injections and from those in the IF-treated group established at 24 h, there was a preponderance of PMNs. A rough quantitation of this observation for each culture is also shown in Table 1. The appearance of the PMNs required the presence of the IF preparation, and their predominance was related to the time of harvest after injection of the samples, being highest shortly after the injection of samples (12 h).

Because of the results from the first and second series of experiments (Fig. 1, 2, 3), it was suspected that NDV and not IF was the cause of

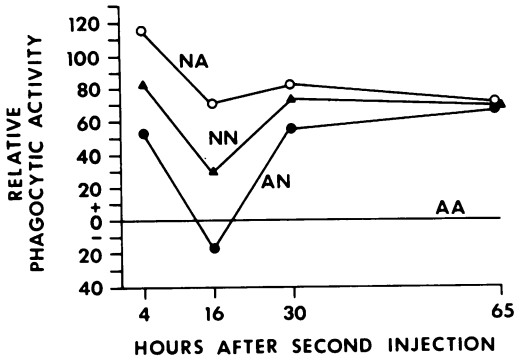

FIG. 3. *Kinetics of the phagocytic response in vivo: a comparison of the effects on mice of injections, given 24 h apart, of combinations of NDV and NAF, NDV and NDV, or NAF and NDV with mice injected with the combination NAF and NAF. Groups of mice received injections of NDV (N) or NAF (A), and 24 h later they received either the same or the reciprocal inoculum. The resulting groups were designated NA, NN, AN, and AA. Phagocytic activity was determined at the indicated intervals after the second injections.*

TABLE 1. *Effect of injection of IF on phagocytic activity and PMN infiltration in the peritoneal cavity of mice*

Sample injected[a]	Hours after injection	% Phagocytic activity/presence of PMNs[b]	Mean phagocytic activity (%)
IF[c]	12	0/2, 0/2, 0/2	0
	24	24/1, 23/0, 17/1	27
	36	40/0, 21/0, 20/0	27
Mock IF[d]	12	4/1, 26/1, 20/0	11
	24	20/0, 23/0, 16/0	20
	36	18/0, 28/0, 22/0	23

[a] Injection of 0.5 ml i.p.
[b] The presence of PMNs was evaluated as 2, 1, and 0 based on the judgment that they were very apparent, apparent, and not apparent, respectively.
[c] IF at 10,000 U.
[d] Concentrated control fluid from C-243 cultures.

the combination of depressed phagocytosis and inflammation seen in this latest experiment. (The IF preparation had routinely been treated with acid to destroy the IF-inducing capacity of the residual virus, but the virus was not removed in this state from the preparation). Therefore, a portion of an IF preparation was centrifuged for 90 min at 105,000 × *g*, and the pellet was suspended to the original volume in EMEM. Another portion was heated at 60 C for 60 min to destroy its IF activity.

Figure 4 depicts the effect these samples had on inflammation in the circulatory system 14 h after 0.8 ml of each was injected i.p. into groups of three mice each. Centrifugation removed the inflammatory principle because the pellet retained activity while the supernatant had lost it. Heat had no significant effect on diminishing the inflammatory properties of the IF preparation.

The inactivation of inducer retained in IF preparations has long been recognized as essential for interpretable antiviral studies. Because even "inactivated" NDV (not capable of inducing IF) had inflammatory properties and by itself could alter phagocytic activity when administered to mice, it was deemed essential to remove it from the IF preparation before studies were conducted on the ability of such a preparation to enhance phagocytic activity in vivo.

In three separate experiments portions of the IF preparation or mock IF used previously were centrifuged at 105,000 × *g* for 90 min before being injected into mice. Twenty-four hours after injection of the samples, blood smears were made from a drop of blood from each mouse, stained and examined microscopically for the presence of PMNs. Phagocytic activity was determined for the same mice 74 h after the injection of samples.

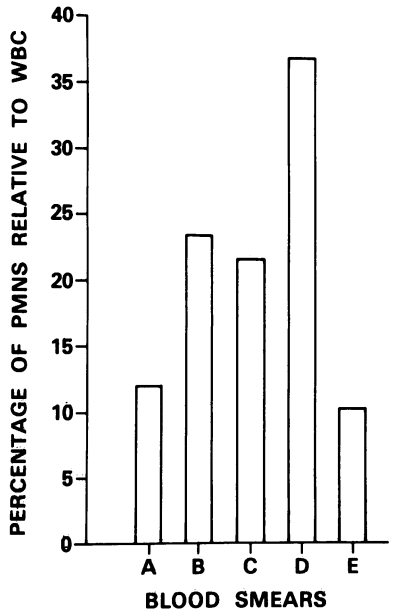

FIG. 4. *Differentiation of an inflammatory principle present in IF preparations from IF. Different physicochemical treatments were performed on an IF preparation. The resultant samples were injected i.p. into mice from which blood was collected and smeared 14 h later. The smears were stained and examined under oil immersion, and the percentage of PMNs relative to the total leukocyte (WBC) count was calculated. (A) IF supernatant after centrifugation for 90 min at 105,000 × g; (B) resuspended pellet after centrifugation of IF for 90 min at 105,000 × g; (C) IF heated for 60 min at 60 C; (D) IF, unprocessed; (E) mock IF.*

In experiment 1, 0.5 ml of either IF or mock IF was injected into two mice each, two times i.p. at 30-min intervals and one time 30 min later subcutaneously. The total amount of IF inoculated was 2.4×10^5 U. In experiment 2, three mice each were injected two times with either IF or mock IF at a 30-min interval (0.5 ml i.p.). The total IF inoculated was 1.6×10^5 U. In experiment 3, 0.5 ml of each preparation, IF, mock IF, or EMEM, was inoculated two times i.p. into eight mice each at a 30-min interval. The total IF inoculated was 1.5×10^5 U.

The results in Table 2 show that the IF preparation significantly enhanced in vivo phagocytic activity. It is also evident that no significant difference in inflammation was induced by the IF preparation in relation to mock IF. Thus, the residual inducer and its accompanying inflammatory activity were eliminated from the IF preparation. Photomicrographs of representative cultures from experiment 2 illustrate the enhanced phagocytosis in response to the IF preparation (Fig. 5).

DISCUSSION

When the data presented above are divided into two areas, the answer to the question of how phagocytosis is affected by IF can be interpreted in two ways.

If phagocytic activity is measured after injection of an IF inducer (NDV), it appears that IF is not involved or at least not the primary source of enhancing activity. A biphasic phagocytic response was observed in mice injected with NDV. Phagocytic activity was depressed around 16 to 18 h, followed by prolonged enhanced activity beginning around 30 h, after the injection of NDV. Characteristic patterns of inflammation were induced by the NDV, as previously described by Borecký and Lackovič (5) and, even under conditions of IF hyporeactivity to induction, no substantial correlation could be made between the amount of IF induced and the amount of phagocytic activity observed. The fact that others have reported similar biphasic kinetics of the phagocytic response of mice after they had injected various inducers of IF other than NDV, such as polyinosinic polycytidylic acid (3), endotoxin (29), and pyran copolymer (A. E. Munson, W. Regelson, W. Lawrence, Jr., and W. R. Wooles, J. Reticuloendothel. Soc. 5:590, 1968) agrees with these findings.

In toto, these observations support the conclusions of Billiau at al. (4), Herman and Baron (13), and Remington and Merigan (24). They showed that protection against various infectious agents was related to enhanced reticuloendothelial activity stimulated by several types of IF inducers. They concluded that the protection was manifest as a nonspecific stimulation caused by the inducer and not by IF itself.

Alternatively, when exogenous IF, produced in cell culture, cleared of inducer, and exposed to highly acidic conditions, was administered to mice, enhancement of their phagocytic activity was observed.

To rectify the apparent dichotomy embodied by these two sets of evidence, it must be realized that it is virtually impossible to demonstrate that IF is solely responsible for enhanced phagocytic activity in vivo after injection of an IF inducer. The reason for this is that IF inducers activate a number of mediators in vivo, along with IF, which could obscure the effects of IF or the ability to measure them. Endogenous pyrogen (1), lymphocytic endogenous mediator (R. S. Pekarek and W. R. Beisel, Fed. Proc. 28:691, 1969), the complement system (22), and

TABLE 2. *Effect of i.p. injection of a high-titer, ultracentrifuged, acid-treated preparation of IF on phagocytosis and inflammation*

Experiment no.	Sample injected	Phagocytic activity[a] (%)		Significance level[b]	Relative PMN count (%)[c]		Significance level
		Individual cultures	Mean		Individual cultures	Mean	
1	IF[d]	32, 48	40	NSD[e]	26, 30	28	NSD
	Mock IF[f]	13, 32	23		26, 33	30	
2	IF[g]	37, 46, NC[h]	42	<0.001	20, 19, 39	26	NSD
	Mock IF	11, 6, 13	10		43, 25, 44	37	
3	IF[i]	42, 41, 40, 39 33, 33, 36, NC	38	<0.001	29, 21, 35, 20 26, 23, 16, 35	26	NSD
	Mock IF	27, 13, 7, 8 10, 11, 25, NC	14		13, 28, 24, 24 22, 20, 18, 26	22	
	EMEM	8, 10, 4, 3, 9 9, 16, NC	8		25, 21, 19, 17 27, 25, 28, 22	23	

[a] Determined 72 h after injection of the samples.
[b] Significance was determined by comparing the effects of IF with mock IF by Student's *t* test.
[c] Determined from blood smears 24 h after injection of the samples.
[d] Injection of 0.5 ml, two times i.p. at 30-min interval; 30 min later, 0.5 ml subcutaneously; total IF = 2.4×10^3 U.
[e] NSD, Not significantly different within a 90% confidence interval or less.
[f] Concentrated control fluid from C-243 cultures.
[g] Injection of 0.5 ml, two times i.p. at 30-min interval; total IF = 1.6×10^5 U.
[h] NC, No carbon; carbon failed to enter peritoneum.
[i] Injection of 0.5 ml, two times i.p. at 30-min interval; total IF = 1.5×10^5 U.

mediators of delayed hypersensitivity (25), to name a few, are factors induced in vivo in concert with IF that could influence the activity of the reticuloendothelial system in a dynamic fashion, if not independently.

Such mediators would not be common in cell culture-derived IF. Still, lymphokines other than IF could be present. Continuous cell lines release MIF spontaneously (21, 28) and after virus infection (10). If spontaneously released MIF were responsible for phagocytosis enhancement, it would have been detected when mock IF was used. The presence of virus-induced MIF or other non-IF lymphokines in the IF preparation was not directly studied. It is not likely that such hypothetical lymphokines would have survived the high acid conditions, pH 2, used to treat the IF preparation in this study. Nonetheless, further physicochemical and biological characterizations with IF preparations should be done to determine whether IF itself or other lymphokines are responsible for phagocytic enhancement in vivo. Tests such as those that identified IF as the probable enhancer of phagocytosis in vitro (9, 14) would be appropriate. Logistically, such tests will be difficult to perform because approximately 10,000-fold more IF is required to produce enhancement of phagocytosis in vivo than in vitro. Ultimately, it will be necessary to obtain pure lymphokines, including IF, to ascertain which factors are responsible for enhancement of phagocytosis.

Thus, because in these experiments an IF preparation was used that contained large amounts of partially physicochemically and biologically defined IF and because of known phagocytosis-enhancing properties of IF in vitro (9, 14), it is probable that IF contributed to the observed enhanced phagocytosis in vivo. This probability is supported by the fact that the IF inducer, NDV, was removed from the IF preparation and could not have played a role in its phagocytosis-enhancing effect.

Evidence is accumulating that IF mediates a number of nonantiviral effects. IF causes IF priming (26) and IF blocking (12), interferes with normal and transformed-cell replication (15) (apparently by altering the rate of deoxyribonucleic acid synthesis [20]), and overcomes the toxic effects of diphtheria toxin on cells (18). It is particularly notable that IF may regulate a number of phenomena involving the immune system. Salvin et al. (25) have shown that type II IF has properties of an MIF. Lindahl-Magnusson et al. (17) have found that IF changes

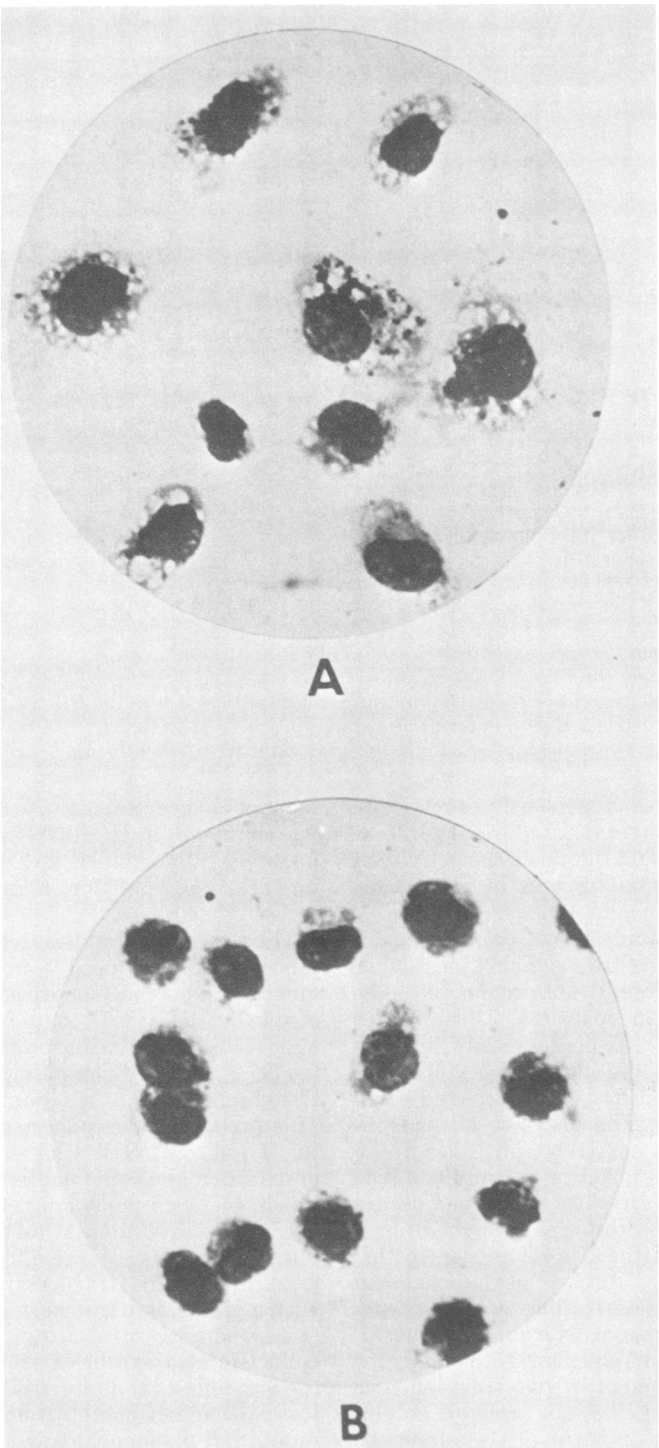

FIG. 5. *Enhanced phagocytosis and concomitant alteration of macrophage morphology seen in photomicrographs of mouse peritoneal macrophages cultures 65 h after injection i.p. of 1.6 × 10^5 U of IF. (A) Cultures from mice that were stimulated by IF; (B) cultures from mice stimulated by mock IF. ×900.*

cell membrane antigenicity and blocks blastogenic transformation (16). Braun and Levy (6) have contended that IF modifies (dose-dependent enhancement or depression) humoral immune responses in vivo. Others have reported similar dichotomous effects on the immune response in vitro (11). A recent report has shown that IF injected into mice blocked, but did not enhance, antibody synthesis (7). Collectively, this evidence supports the concept that IF can enhance phagocytosis in vivo.

The role of IF in all nonantiviral activities may be doubted as long as pure IF is unavailable for experimentation. Nonetheless, the fact that IF preparations enhance phagocytosis in vivo should interest students of IF and lymphokines alike.

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