

# Production of Macrophage Migration Inhibition Factors by Virus-Infected Cell Cultures

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Macrophage migration inhibitory (MIF-like) activity was demonstrated in the supernatant fluids from cultures of African green monkey kidney cells (BGM) infected with mumps virus or Newcastle disease virus. We could detect no such activity in noninfected cultures. The virus-induced activity reported here is not due to nonspecific cytotoxic material released by dead or dying cells, and it does not require cell replication for its production. Preliminary estimates of molecular weight by Sephadex G-100 chromatography revealed a broad band of activity associated with the 45,000 and 65,000 markers. These are significantly smaller than previously reported chemotactic substances from virus-infected cultures, and thus appear to represent different cell products. These MIF-like factors may be produced concomitantly with interferon. However, ultraviolet irradiation of appropriate duration abolishes the ability of viruses to induce substances with MIF-like activity while preserving the ability to induce interferon. This strongly suggests that interferon is not the agent responsible for the macrophage migration inhibition effect. The functional properties of these various cell products induced by virus infection suggest that they all may play a role in the response to virus infection *in vivo*.

The interaction between specifically sensitized lymphocytes and antigen *in vitro* leads to the production of a factor (MIF) which inhibits the migration of macrophages (9, 6). This property is a function of the state of delayed hypersensitivity of the lymphocyte donor (5, 13). It is also possible to obtain migration inhibitory activity from lymphocytes in the absence of an immune reaction; this can be accomplished by stimulating normal lymphocytes with such substances as phytohemagglutinin (12), concanavalin A (12), and staphylococcal enterotoxin B (10). Recent reports have also described MIF or MIF-like activity in supernatant fluids of proliferating lymphocyte cultures in the absence of mitogenic stimulation and similar activity in supernatant fluids from cultures of various nonlymphoid cells such as fibroblasts derived from lung and brain (11, 16). It therefore appears as though substances which inhibit macrophage migration may be widely distributed in nature, although it is likely that the mechanisms by which these substances exert their effect may vary. Cells may be triggered to synthesis and/or release of these materials by a variety of inciting agents of which antigen is only one example.

MIF is one of a number of substances produced by antigen-activated lymphocytes which are collectively known as lymphokines. Several lymphokines with leukotactic activity have also been described (18). Recently, it has been reported (17) that virus infection of nonlymphoid cell lines results in the production of leukotactic factors as well. In the light of this observation and the available information on the ubiquity of substances which inhibit macrophage migration, we felt it important to explore the ability of virus-infected cells to produce factors with MIF-like activity. Such factors, regardless of whether or not they prove to be identical with classic MIF, may play a role in defense mechanisms in viral disease.

## MATERIALS AND METHODS

**Cell lines.** The BGM line of African green monkey kidney cells (2) was used in this investigation. Cells were grown in Eagle minimal essential medium in Earle saline supplemented with 10% fetal calf serum and 100 U of penicillin per ml and 100 µg of streptomycin per ml. Maintenance medium for virus experiments was medium 199 with 2% fetal calf serum and the above antibiotics. Cultures used in these experiments were grown in 4-ounce prescription bottles (ca. 119 ml) containing 10 ml of medium.

**Virus preparations.** The ABC strain of mumps virus (MV) was used. Seed virus was prepared as culture fluid from the seventh passage in primary rhesus monkey cell culture. The pool titered  $3.0 \times 10^7$  plaque-forming units (PFU) per ml in BGM cells. The Victoria strain of Newcastle disease virus (NDV) was prepared as infectious allantoic fluid titering  $10^{9.3}$  embryo lethal doses 50% per ml. The agent had been passaged 10 times in chicken embryos. The Indiana strain of vesicular stomatitis virus (VSV) was used for interferon assay. The preparation was allantoic fluid which titered  $1.7 \times 10^9$  PFU/ml in BGM cells.

**Infectivity assays.** Harvested supernatant fluids from MV-infected cell cultures were assayed for infectious virus by plaque formation in BGM cells (8). NDV was assayed by inoculation of 9- to 10-day old embryonated chicken eggs by the allantoic route and observation for embryo death.

**Interferon assay.** Interferon from the BGM cultures was assayed by an adaptation of the microtiter technique described by Armstrong (1). BGM cells were grown in 96-well plastic plates (Linbro Chemical Co., New Haven, Conn.). Putative interferon preparations were diluted in fourfold increments in medium 199, 2% fetal calf serum, penicillin, (100 U/ml) and streptomycin (100  $\mu$ g/ml). Interferon dilutions were placed in the wells with the BGM monolayers, four replicates per dilution, incubated overnight at 36 C, and challenged with 150 to 300 PFU of VSV per well. Plates were further incubated for 48 h and then stained with methyl violet (0.5% methylrosaniline, 5.0% formaldehyde, 50% ethyl alcohol, and 0.85% NaCl in water). Cell protection was assessed by comparison with uninfected cell controls (4+ protection) and complete cell destruction (0 protection) in unprotected virus-infected controls. An internal laboratory standard preparation of monkey interferon was run as an interferon control. Interferon titers were expressed as the reciprocal of the highest dilution of material which resulted in a calculated average 2+ protection.

Before testing for interferon activity, fluids were dialyzed against 0.85% NaCl at pH 2.0 for 24 h at 4 C, then against Earle saline, pH 7.2, for 24 h at 4 C. Fluids were centrifuged at  $98,000 \times g$  for 1 h.

**MIF assay.** Fluids to be tested for MIF activity were removed from monolayers and ultracentrifuged at  $98,000 \times g$  for 1 h to sediment virus particles. The supernatant fluids were not concentrated before the study. They were assayed for MIF with the following method. Normal Hartley guinea pigs received an intraperitoneal injection of 20 ml of sterile light mineral oil. Four days later, the peritoneal cavity was washed out with sterile Hanks balanced salt solution, and the cells were collected by centrifugation at  $900 \times g$  for 5 min, washed twice, and suspended in RPMI 1640 supplemented with 10% normal guinea pig serum. Capillary tubes containing cells were prepared and cultured for 24 h according to Yoshida's modification of David's methods (21). Each test fluid was assayed at least twice. For each assay, at least four capillary tubes were evaluated. Determination of the area of migration and the statistical treatment of the data were carried out as described previously (19).

The migration index expressed the migration area of the experimental preparation as a percentage of the migration of the control.

In these experiments, in which the effect of virus infection was studied, the control was maintenance medium taken from uninfected cultures of similar duration and incubated under similar conditions, rather than fresh medium.

**Viability studies.** Any factor which is cytotoxic for macrophages will obviously inhibit migration. To rule out this possibility, two additional procedures were performed. In some experiments, after measurement of migration area, the migration cells were recovered from the chambers and viability was ascertained by trypan blue exclusion. In other experiments, the migration chambers were allowed to remain in culture for 72 h to demonstrate reversibility of migration inhibition.

**Fractionation of MIF-rich fluids.** Lyophilized supernatant fluids equivalent to 100 to 150 ml of starting material were redissolved in 2 to 3 ml of buffer (0.01 M phosphate-buffered saline, pH 7.4), clarified by centrifugation, and applied to a column (2.5 by 40 cm) of Sephadex G-100 equilibrated with buffer. The eluate was collected at a flow rate of 0.1 ml/min in 3.0-ml samples. Effluent fractions near the void volume (which contain proteins of high molecular weight) were pooled as fraction I. Fractions containing the phenol red marker were pooled as fraction VI. Intermediate fractions of equal volume were pooled as fraction II-V (approximately 30 ml per fraction) and lyophilized. They were redissolved into 3 ml of buffer and dialyzed against RPMI 1640 medium before use. Each fraction was tested for MIF activity.

## RESULTS

**Background activity.** Previous investigators have demonstrated MIF-like activity in uninfected cell culture fluids. We therefore examined our culture supernatant fluids for such activity before inoculation. The culture fluids from BGM cells after 24 and 48 h of incubation with maintenance medium were negative, with indices of  $101.0 \pm 9.7$  and  $95.1 \pm 9.3$ , respectively. The control for this experiment was fresh medium 199 with 2% fetal calf serum and antibiotics in the same concentration as in the test medium. These cultures were complete monolayers at the start of the incubation periods.

**Supernatant activity of infected cultures.** Cultures of BGM cells were infected with either MV or NDV by inoculation of 0.1 ml of seed virus preparation and incubation at 37 C. Supernatant fluids were sampled at 24 and 48 h after inoculation. Table 1 shows results at 48 h, when peak activities were obtained. In both cases, virus infection resulted in significant activity, with migration indices of 56.1 and 60.9. As stated previously, in this experiment the migration indices were calculated by comparing

TABLE 1. MIF activity in fluids from virus-infected BGM cell cultures

Material tested	Migration index <sup>d</sup>
Mumps virus culture fluid <sup>a</sup> . . . . .	56.1 ± 3.1
Newcastle disease virus culture fluid <sup>b</sup> . . . . .	60.9 ± 2.9
Freeze-thawed extract <sup>c</sup> . . . . .	108.1 ± 9.1
Uninfected cell culture fluid . . . . .	(100.0)

<sup>a</sup> Collected from BGM cell cultures 48 h after infection with mumps virus ( $3.0 \times 10^6$  PFU).

<sup>b</sup> Collected from BGM cell cultures 48 h after infection with Newcastle disease virus ( $10^{8.3}$  embryo lethal doses 50%).

<sup>c</sup> Supernatant fluid from cell suspension subjected to three cycles of freezing and thawing.

<sup>d</sup> Calculated as (mean area of test fluid migration/mean area of uninfected control fluid migration) × 100.

the area of migration of macrophages incubated with the culture fluids from infected cells to the area of migration of macrophages in culture fluid from uninoculated cells incubated for similar periods of time. This is a more rigorous control than the use of fresh medium, and was adhered to in all the experiments which follow in spite of our failure to detect background activity in the uninfected cultures. As an additional control, supernatant fluids from three cycles of freezing and thawing of uninfected cells did not contain substances which inhibit macrophage migration (Table 1).

The supernatant fluids from infected cultures were subjected to centrifugation of sufficient duration and intensity ( $98,000 \times g$  for 1 h) to remove virus particles. This was confirmed by demonstrating lack of infectivity or hemagglutinating activity in the supernatant fluids.

Studies of Sephadex fractions of culture fluids from MV- and NDV-infected cells showed peaks of migration inhibitory activity in fractions II and III as previously defined. These corresponded to an approximate molecular weight of between 65,000 and 45,000. The migration indices obtained were  $65.3 \pm 10.5$  for fraction II and  $48.9 \pm 10.2$  for fraction III. In these experiments, the control consisted of corresponding fractions of uninfected material.

**Viability studies.** It is important to exclude cytotoxicity as the basis for the observed macrophage migration inhibition in these studies. Accordingly, after measurement of migration area, we recovered the migrated cells from the chambers by gentle pipetting and added 0.4% trypan blue. By the criterion of trypan blue exclusion, the viability of the cells from control preparation was 76%. The positive preparations

(with average migration index of 59.2) had essentially identical viability counts (75%).

To further explore this point, we allowed some migration chambers to remain in incubation for 72 h and determined the migration patterns at various times. In a typical experiment with fluid from NDV-infected BGM cultures, the migration index at 24 h was  $63.8 \pm 3.4$ . At 48 h the index had risen to  $73.2 \pm 2.8$ , and by 72 h the index was  $107.9 \pm 4.4$ . Similar results were obtained for the other positive preparations. This reversibility of migration inhibition demonstrated that the results were not due to cytotoxic effects.

**Viral growth.** The growth of MV and NDV in cultures of BGM cells is described in Fig. 1. Both viruses replicated; however, MV grew to two  $\log_{10}$  increments greater in titer within the 48 h of the experiment.

**Production of MIF-like activity and interferon.** Table 2 shows the appearance of MIF activity in relation to interferon production which accompanied the growth of the viruses in the cells. In previous experiments, the ratio of infectious virus particles to cells was 1,000 for NDV and 10 for MV. In this experiment the ratios were both 0.01; therefore, replication of the agents was required to provide sufficient stimulation of the culture to produce detectable amounts of MIF and interferon. The appear-

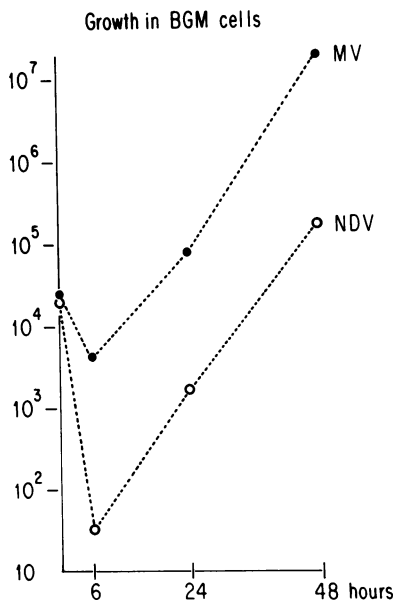


FIG. 1. Growth of mumps virus (MV; ●) and Newcastle disease virus (NDV; ○) in BGM cultures. (MV titers as PFU/ml, NDV titers as embryo lethal doses 50%/ml).

TABLE 2. *MIF and interferon activity in culture fluid from mumps virus- and Newcastle disease virus-infected BGM cells*

Time (post-inoculation)	Mumps virus <sup>a</sup>		Newcastle disease virus <sup>b</sup>	
	Migration index	Interferon activity	Migration index	Interferon activity
2 h	N.D. <sup>c</sup>	<4	101.2 ± 2.5	<4
6 h	N.D. <sup>c</sup>	<4	108.1 ± 2.0	<4
24 h	71.1 ± 4.9	<4	98.7 ± 2.7	<4
48 h	56.1 ± 3.1	64	59.5 ± 3.5	134

<sup>a</sup> BGM cell cultures inoculated with  $2.4 \times 10^4$  PFU of mumps virus.

<sup>b</sup> BGM cell cultures inoculated with  $10^{4.3}$  embryo lethal doses 50% of Newcastle disease virus.

<sup>c</sup> N.D., not done.

ance of MIF in MV-infected cultures preceded the detection of interferon, whereas both MIF activity and interferon were detected concomitantly in the NDV-infected cultures.

To further study the relationship between these two activities, we performed viral inactivation studies. NDV seed virus was inactivated by ultraviolet irradiation for various periods of time. Samples of virus from each period were titrated for infectivity, and 0.1 ml of each was inoculated into BGM cell cultures. Supernatant fluids were harvested after 24 h of incubation and assayed for MIF and interferon (Table 3). Irradiation of the inoculum for 30 s reduced the infectivity approximately two log<sub>10</sub> increments yet did not affect the ability of the virus to induce MIF or interferon. Longer irradiation resulted in loss of the capacity to stimulate MIF, yet these inocula were still capable of evoking the production of interferon. Therefore, although fully infectious virus is not necessary to stimulate MIF, the requirement of the stimulating virus for the interferon response and the MIF response appear to be different.

## DISCUSSION

Sensitized lymphocytes, when stimulated by specific antigen, in vitro release various substances into the culture media which are known as lymphokines (7). These include MIF, lymphotoxic factor, blastogenic factor, chemotactic factors, and interferon, as well as other substances with biologic properties which may play a role in manifestations of cellular immunity in vivo. Nonimmunological stimulation of lymphocytes by mitogens such as phytohemagglutinin or concanavalin A can also lead to lymphokine production. Moreover, as stated above, certain established cell lines, both

TABLE 3. *Effect of ultraviolet light inactivation of Newcastle disease virus on production of MIF and interferon in BGM cells*

Time of irradiation <sup>a</sup> (s)	Infectivity titer	Migration index	Interferon activity
0	10 <sup>7.7</sup>	79.2 ± 4.1	1,240
30	10 <sup>6.8</sup>	74.7 ± 8.1	460
60	10 <sup>4.8</sup>	97.7 ± 5.9	460
120	10 <sup>5.2</sup>	93.3 ± 6.0	476

<sup>a</sup> Virus irradiated 20 cm from the ultraviolet light source (Westinghouse Sterilamp 782L-30).

lymphoid and nonlymphoid in nature, have been shown to release similar factors in the absence of apparent stimulation. Therefore, it appears that these effector substances may have a more general role than the mediation of immune reactions. We have recently shown that virus infection of cell lines in culture is an adequate stimulus for the release of chemotactic factors into the medium (17). These factors are functionally equivalent, but they are not identical with similar substances produced by antigen-activated lymphocytes. In the present study, we show that certain in vitro virus infections can trigger the release of substances which inhibit macrophage migration in vitro. This inhibition is not due to the presence of cytotoxic substances, because there is no difference in viability, as determined by trypan blue exclusion, between positive and control fluids, and the migration inhibition is reversible after prolonged incubation of the migration chambers. The factor which produces this MIF-type macrophage migration inhibition does not appear to represent preformed material released by cells dying as a consequence of viral infection; mechanical disruption of BGM cells does not lead to extract fluids with such activity. As was the case for virus-induced macrophage and neutrophil chemotactic factors (17), the activity is not a property of the virus itself. By these various criteria, the factor involved is MIF-like. However, no attempt was made in these studies to establish their identity with conventional MIF derived from antigen-activated lymphocytes, because it is likely that even this latter material represents several molecular species with similar biological activity. In the present study, supernatant fluids derived from infection of BGM cells with MV and NDV were subjected to column chromatography, and activity was obtained in fractions corresponding to molecular weights between 45,000 and 65,000. These values approximate those obtained for molecules with MIF activity derived

from other sources (21) and for MIF itself in some reports (4), although they are different from the value (23,000) obtained for human MIF by Rocklin et al. (14).

It should be emphasized that the MIF-like activity reported here is a specific consequence of virus infection. Background activity (11, 16), which appears as a consequence of incubation and proliferation in the absence of known virus infection, was not detected in the BGM cell line. We did not examine supernatant fluids from actively proliferating BGM cultures, however. Tubergen and colleagues (16) have explored the relationship between spontaneous elaboration of MIF in replicating cultures and the cell replicative cycle. They conclude that MIF is released upon entry of a cell into the S phase and that the significance of MIF in delayed hypersensitivity lies in its relation to cellular proliferation rather than a direct relationship to immunologic specificity.

On the other hand, Bloom et al. (3) and Rocklin and Ratcliffe (Fed. Proc. 31:3029, 1972) demonstrated, with the aid of inhibitors of mitosis and deoxyribonucleic acid synthesis, that antigen-induced MIF in the guinea pig may be produced by nondividing cells. It is significant that in our studies, virus-induced production of MIF was detected in contact-inhibited cultures. This would suggest that the production of these substances is not restricted to replicating cells but can result from other appropriate stimuli such as virus infection as well as antigen stimulation. This observation does not preclude the possibility that some common physiological state is required for production of MIF activity and that this state may be a consequence of either passage through a replicative cycle or of various kinds of stimulation.

In these studies, the cell concentration during incubation with virus were of the order of  $5 \times 10^5$  per culture. This is approximately 10% of that used for production of MIF by antigen-stimulated lymphocytes. Because we never required concentration of the supernatant fluids, this suggests that the virus system is more efficient. The inefficiency of antigen-induced MIF release is probably due to the restricted population of lymphocytes which can respond to a given antigen.

Although direct evidence is lacking, it does not seem that the various biological activities detected in the supernatant fluids of virus-infected cell cultures are properties of the same molecule. The data from the ultraviolet studies show that inactivated virus may retain its ability to stimulate interferon activity, al-

though it is rendered incapable of evoking the production of the MIF-like factors. Sucrose density gradient studies (17) of the virus-induced chemotactic factors previously described showed that this activity sedimented with the immunoglobulin marker, suggesting a much larger molecule than most reported estimates of interferon size, as well as one that is larger than the factors described in this report.

Salvin et al. (15) have recently reported the induction of circulatory MIF and interferon by challenge of BCG-sensitized mice with old tuberculin. These activities were demonstrable in plasma and were not separable by Sephadex G-100 filtration. In addition, both activities were destroyed by acid conditions (pH 2.0). Inoculation of NDV into mice resulted in stimulation of interferon without accompanying MIF activity, and the interferon was stable at pH 2.0. In our study, we were able to observe interferon in the absence of migration inhibition and vice versa. The interferon was stable at pH 2.0. It is interesting to note that the MIF reported by Salvin et al. had gel filtration characteristics similar to that of the preparations we have studied; both were found in fractions with molecular weight markers of approximately 40,000 to 80,000.

The feature common to both chemotactic factors and the MIF-like factor which can be generated by in vitro virus infection is that they have biological effects on inflammatory cells. In the case of the former, in vivo generation has been shown as well by using chicken chorioallantoic membrane as a source of infected cells. If the production of the factors reported here can also be demonstrated in an in vivo system, it will suggest that this represents a general phenomenon which might form the basis of a previously undefined defense system in viral disease.

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