LEUKOTACTIC FACTORS ELABORATED BY VIRUS-INFECTED TISSUES*

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Many virus infections are associated with an inflammatory response characterized by more or less homogenous accumulations of inflammatory cells. For example, in poliomyelitis focal collections of neutrophils are found in association with infected and damaged anterior horn motor neurons. On the other hand, many meningoencephalitis-inducing viruses give rise to infections characterized by predominantly lymphocytic and monocytic infiltrations. There is very little information regarding the factors that promote the accumulation of one or the other of the various inflammatory cell types at sites of virus infection.

One approach which has proved useful in the study of various kinds of inflammatory responses has been the investigation of various factors which cause specific, unidirectional migration (chemotaxis) of inflammatory cells. Most of the studies along these lines have made use of the Boyden technique, which allows for quantitative measurements of chemotaxis in vitro (1, 2). By this technique, many leukotactic factors have been discovered. Factors have been found which are active for neutrophils (3–5), eosinophils (6, 7), macrophages (8, 9), and lymphocytes (3). Some of the chemotactic factors are active for more than one type of inflammatory cell, while others appear to be cell specific. Some of the factors described are derived from substances endogenous to the experimental animal under study; for the most part these are products of complement activation or substances released by antigen-activated, sensitized immunocompetent cells. The complement-derived factors are usually released as a consequence of activation of the complement system, resulting in alteration and/or cleavage of complement components by enzymes intrinsic to the complement system. Recently, it has been shown that certain mammalian cells contain substances that can cleave the third or the fifth components of complement productive of chemotactic peptides (10–12). These substances may represent enzymes. Many other enzymes extrinsic to the complement system can likewise generate similar or identical chemotactic factors (13, 14).

Leukotactic factors may also be derived from endogenous materials, for example,

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the substance obtained from filtrates of *Escherichia coli* cultures. This material is chemotactic for neutrophils and, to a lesser extent, for eosinophils (7, 13). Virus particles themselves have not been shown to possess such activity. However, infection of tissue cultures with herpes simplex virus results in the release of a factor which generates from C5 a leukotactic product (12). Similar leukotactic factor-generating activity may be obtained from lysates of uninfected cultures (12). Thus, this generator of leukotactic activity exists in normal cells and may represent a lysosomal enzyme.

The data to be presented in this paper will show that when chicken embryos or monkey kidney-derived tissue cultures are infected with mumps (MV)[1] or Newcastle disease virus (NDV), chemotactic activity for neutrophils and macrophages can be detected in the various supernatant fluids. The leukotactic material described here is not obtainable from uninfected materials and does not discernibly depend upon complement components for activity.

Materials and Methods

Virus Preparation.—A preparation of the Victoria strain of NDV ($10^{9.3}$ ELD_{50}/ml was used. 9-day chick embryos were inoculated with $10^{6.3}$ ELD_{50} of NDV and incubated at 35°C; the allantoic fluid was harvested 48 hr later. MV strains used were the chick embryo-adapted Enders strain ($10^{8.6}$ EID_{50}/ml) and the mammalian cell-adapted ABC strain (1.2×10^7 plaque-forming units/ml). 8-day chick embryos were inoculated with $10^{5.6}$ EID_{50} of Enders strain virus and incubated at 35°C; the allantoic fluid was harvested 120 hr later. ABC virus was inoculated into monolayer cultures of BGM cells, a continuous line of African green monkey kidney cells (15). Cultures were maintained with Medium 199 containing 2% fetal calf serum penicillin (100 units/ml), and streptomycin (100 μg/ml). Culture fluids were harvested after 48 hr incubation at 37°C.

Leukotactic Assays.—A modified Boyden chamber technique was used. This procedure, which has been described in detail (16), involves the separation of a chamber into an upper compartment containing leukocytes, and a lower compartment containing the putative leukotactic substance. The two compartments are separated by a micropore filter. Migration of cells through the pores of the filter in excess of that observed in control chambers, which either lack leukotactic substance or contain the same concentration of the substance in both chambers (to abolish the gradient), is taken as evidence of leukotactic activity. In the present experiments, indicator cells consisted of rabbit neutrophils obtained by the intraperitoneal injection of 0.1% glycogen in saline 4 hr previously (10) or rabbit macrophages obtained 4 days after the intraperitoneal injection of mineral oil (8). All cells were suspended in Medium 199 with 10% homologous serum. In several experiments it was shown that suspension of cells in 0.1% bovine serum albumin gave similar results, indicating that serum was not required in the cell suspension for the detection of chemotactic activity. Unless otherwise indicated, 100 μl of the various substances tested for chemotactic activity was added to appropriate compartments. Cell migration was quantitated by counting the number of migrating cells in five high-power fields under a light microscope. When neutrophils were used, filters of pore size 0.65 μ were used in the chambers; with macrophages 5 μ pore filters were utilized.

Centrifugation Studies.—Ultracentrifugation was performed in a Beckman L2-65 ultracentrifuge (Beckman Instruments Inc., Fullerton, Calif.) using a swinging bucket rotor at 45,000 rpm (200,000 *g*) for 15 hr at 4°C. Sucrose gradients from 7.5 to 35% were made up in phosphate buffer, pH 7.3, ionic strength 0.05. Details of this procedure have been previously described (16).

[1] *Abbreviations used in this paper*: MV, mumps; NDV, Newcastle disease virus.

Complement Components.—The third (C3) and the fifth (C5) components of human complement were isolated according to the method of Nilsson and Müller-Eberhard (17). These individual complement components were mixed with the various culture fluids and appropriate volumes of phosphate-buffered saline (pH 7.4) in a total volume of 0.25 ml and incubated for the indicated period of time at 37°C, and the mixtures were then diluted to 1.0 ml with Medium 199. These fluids were subsequently tested for leukotactic activity. By this procedure it is possible to determine if a culture fluid contains a material, such as an enzyme, that will generate leukotactic activity from added complement components (18).

RESULTS

Newcastle Disease Virus Infection.—Allantoic fluids from NDV-infected chicken embryos were assayed for leukotactic activity. As indicated in Table I, such fluids contained leukotactic activity for neutrophils in each of four different experiments, while the control fluids from embryos inoculated with

TABLE I

Neutrophil Chemotactic Activity in NDV-Infected Allantoic Fluid

Material tested	Leukotactic activity*			
	Exp. A	B	C	D
100 µl				
Uninfected allantoic fluid	10	40	0	15
Infected allantoic fluid	115	145	50	190
Infected allantoic fluid, ultracentrifuged:				
Supernate	—	—	45	155
Pellet	—	—	20	50
Medium 199	0	0	0	0

* Sample volumes of 100 µl tested.

virus-free diluent (phosphate-buffered saline) showed little or no activity. In order to define the nature of the leukotactic activity, two fluids (Table I, exps. C and D) were submitted to centrifugal forces of 200,000 *g* for 4 hr. The supernates and pellets were then obtained, the latter being resuspended in Medium 199 to the starting volume. Leukotactic activity was assessed in each preparation and found to be present predominantly in the supernate fractions (Table I, exps. C and D). The lower, but definite, level of activity in the pellets was probably due to a carry-over of supernatant fluid with the pellet in the centrifuge tube, since pellets were not washed before resuspension. These results suggest that the leukotactic activity in the infected fluids is associated not with intact virus particles but, rather, with a nonsedimenting material.

Dose responses for fluids from NDV-infected embryos were determined (Fig. 1). Fluids A and B, from two different experiments, showed similar dose-response relationships with a direct correlation between volume of the fluid tested and the amount of the leukotactic activity. One fluid (B) was also centrifuged (as above), and the supernate was compared with the original infected fluid

(Fig. 1). That both lines are nearly congruent suggests that removal of the virus by ultracentrifugation does not alter the concentration of leukotactic activity in the fluid. This supports the contention that leukotactic activity is not associated with virus particles per se.

Macrophage Chemotactic Activity.—We next attempted to explore chemotactic activity for mononuclear cells as well as neutrophils. The indicator cells for these experiments were macrophages from peritoneal exudates, obtained as described above. Chicken embryos were infected with either NDV or Enders strain MV. The results are shown in Table II. Significant levels of macrophage chemotactic activity were present in both preparations. These fluids were also assayed for neutrophil activity and found to be positive, with results quantita-

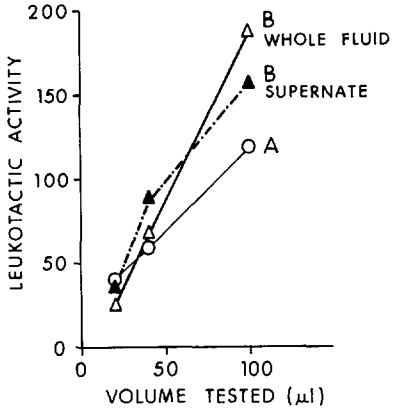

FIG. 1. Dose responses of allantoic fluids from NDV-infected chick embryos. In both experiments A and B, the dose response curves are linear. "B Supernate" is fluid B ultracentrifuged to remove viral particles.

tively similar to those of the previous experiment (Table I). In comparison, the MV-infected material was over twice as effective for macrophage, as compared with the NDV-infected material. It is not known if the leukotactic activity in these preparations consists of two factors, each uniquely active for one type of leukocyte (3), or if there is one factor with activity for both types of leukocytes (8). Supernates from freeze-thawed uninfected chorioallantoic membranes were devoid of either type of activity, as was allantoic fluid from intact embryos inoculated with diluent alone.

Tissue Culture Studies.—Fluids from ABC MV-infected BGM cell cultures were assayed for leukotactic activity. These results are seen in Table III. Again, chemotactic activity for both neutrophils and macrophages was detected. Supernates of freeze-thawed cultures were negative for chemotactic activity, suggesting that nonspecific cell injury does not lead to the release of preformed leukotactic factors residing normally in cells before infection.

Density Gradient Analysis of the Virus-Stimulated Leukotactic Factors.—The chemotactic activity in fluids from chicken embryos infected with NDV or MV and in fluid from MV-infected cell cultures was studied by density gradient centrifugation. Chemotactic activity for neutrophils in the NDV-infected allantoic fluid was biphasic; one peak sedimented near the IgG marker and the second sedimented near the cytochrome *c* marker (Fig. 2, *upper frame*). Two similar zones of activity were noted in allantoic fluid from chick embryos infected with MV (Fig. 2, *lower frame*). On the other hand, fluid from a cell culture

TABLE II

Macrophage and Neutrophil Chemotactic Activity in NDV- or MV-Infected Allantoic Fluids

Materia :tested	Chemotactic activity	
	Macrophage	Neutrophil
150 µl		
Allantoic fluid, diluent inoculated	0	0
Allantoic fluid, NDV infected	95	120
Allantoic fluid, MV infected	260	145
Supernate, freeze-thawed chorioallantoic membrane	0	0

TABLE III

*Macrophage and Neutrophil Chemotactic Activity in MV-Infected Cell Culture Fluids**

Material tested	Chemotactic activity	
	Macrophages	Neutrophils
150 ml		
Culture fluid, uninfected	0	20
Culture fluid, MV infected	170	150
Supernate, freeze-thawed culture cells	0	0
Medium 199	0	0

* BGM cells maintained in Medium 199 as defined in text.

infected with MV showed activity that was limited to a zone coincident with the IgG marker (Fig. 2, *middle frame*). These studies indicate a similarity in distribution of chemotactic factors in allantoic fluids infected with two different viruses. Conversely, the same virus (MV) grown in two different systems (embryo vs. cell culture) led to a different ultracentrifugal distribution of leukotactic activity in the culture fluids.

Complement-Derived Leukotactic Activity.—Brier et al. (12) have reported that tissue culture fluid from rabbit kidney monolayers infected with herpes simplex virus will release a material capable of generating a C5-dependent factor chemotactic for neutrophils. We tried to determine if the fluids used in the present studies had similar complement-dependent leukotactic factor–gen-

erating activity in addition to the chemotactic activity described above. In these experiments, 5 μl volumes of various fluids (an amount containing no detectable leukotactic activity) were added to purified preparations of human C3 or C5 (20 μg each). The mixture was incubated at 37°C for 10 min and then assayed for chemotactic activity. The results are shown in Table IV. Fluids

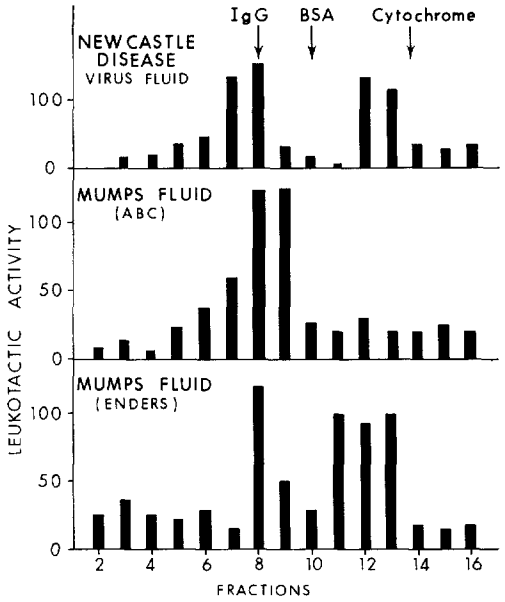

FIG. 2. Ultracentrifugation analysis in sucrose density gradients of chemotactic activity in fluids from virus-infected supernates (allantoic fluid, *upper and lower frames*) and BGM cell culture fluid (*middle frame*). This direction of sedimentation is from right to left. Protein standards are indicated: IgC (human), bovine serum albumin (BSA), and cytochrome *c*.

TABLE IV

Generation of Neutrophil Chemotactic Activity by Addition of Human C3 or C5 to Culture Fluids of Mumps and Newcastle Disease Viruses

Material incubated with complement*	Leukotactic activity generated from	
	C3	C5
Allantoic fluid, uninfected	0	0
Allantoic fluid, NDV infected	35	110
Allantoic fluid, MV infected	85	110
MV-infected cell culture fluid	0	20
Cell culture fluid, uninfected	0	25

* Reaction conditions: 5 μl of fluid + 20 μg of complement protein (in 50 μl), with phosphate-buffered saline (pH 7.4) added to give a volume of 200 μl. The mixture was then incubated at 37°C for 10 min.

from chicken embryos infected with either NDV or MV were able to generate leukotactic activity in the presence of either C3 or C5, although substantially more activity was generated from C5. Curiously, little or not activity could be generated from either complement component when fluid from MV-infected BGM cell cultures was used.

DISCUSSION

Induction of the inflammatory response in tissues infected by viruses may be associated with the elaboration of chemotactic factors that cause the accumulation of various kinds of inflammatory cells. These tissue-dependent chemotactic factors fall into two distinct categories: those that are fully formed and those that require interaction with serum factors. In the present studies, viruses appear to cause the elaboration of "complete" leukotactic factors, that is, no additional interaction between virus and/or virus product and serum or complement is required. This is somewhat similar to the case of bacteria which, in the presence of replication, release leukotactic factors of high ($>20,000$) and low ($<10,000$) molecular weights (14, 19). These factors are fully active and independent of serum factors, although it should be pointed out that same bacteria also release generators (C3 and C5 dependent) of leukotactic activity. Within experimental limits, the data presented in this paper (Table II) suggest that the leukotactic factor(s) elaborated by virus infection of chicken embryo or BGM cell cultures are not products that can be detected in uninfected cells. Whether the factors described in the present study are synthesized or are *de novo* produced from precursor substances in cells cannot be determined from the data presented.

As shown by the density gradient studies, Table I, Fig. 1, the leukotactic factors obtained from either infected chicken embryos or BGM cell cultures do not appear to be associated with intact virus particles. As seen in (Fig. 2) ultracentrifugally simiar leukotactic factors are released into allantoic fluid during infection with two different viruses. This suggests that production of the leukotactic factors is a function of properties of the infected cell rather than the particular infecting virus. There are, of course, other possible explanations. The more limited pattern of leukotactic activity in the infected BGM cultures may reflect a greater homogeneity of cell population in these cultures as compared with the chicken embryo. An additional possibility must be considered: the presence in the embryo of C5 could furnish the substrate for a C5-cleaving enzyme.

It is known that leukotactic factors may be produced by tissue damage. This depends on the release of a proteolytic enzyme. Hurley (20) found that disrupted normal tissue incubated with serum resulted in generation of neutrophil leukotactic activity. Hill and Ward (10) showed that this was due to the cleavage of C3 by a neutral protease, naturally residing in many tissues and released under the conditions of anoxia (21). Neutrophilic granulocytes contain within

their lysosomal enzymes a neutral protease capable of cleaving a leukotactically active peptide from C5 (11). This enzyme can be released under the influence of phagocytic events in the neutrophil (11). A similar C5-cleaving enzyme has been found in the kidney cells and its release has been documented by Brier et al. (12) in the case of infection with the herpes simplex virus, as pointed out above. The present studies extend these findings and indicate that C3- and C5-dependent leukotactic factor–generating enzymes can be released from chicken embryos or BGM cell cultures, as a consequence of infection with two different viruses. The predominant factor released that has ability to generate leukotactic activity is the C5-dependent generator of leukotactic activity. Of course, the release of these leukotactic factor generators may simply be secondary to virus-induced cell injury. This is in contrast to the leukotactic factors, defined in the present study, that do not require an exogenous complement source, and which cannot be obtained from lysates of uninfected cells.

The generation of leukotactic activity for both neutrophils and macrophages in the course of virus infection may be analogous to the production of interferon and, if it occurs in vivo, may similarly serve a protective role. In this regard, although the relationship between elaboration of interferon activity and leukotactic activity has not been determined in these experiments, it is likely that the two activities are not properties of the same molecule. Most of our activity was associated with material whose behavior in the ultracentrifuge suggests a molecular weight of approximately 150,000, which is higher than any reported value for interferon. Also interferon activity is species specific, whereas the factors described here were obtained from chicken or monkey cells and were active against inflammatory cells of rabbit origin.

SUMMARY

Infection of chick embryos wih either Newcastle disease virus or mumps virus and infection of BGM cell cultures with mumps virus result in the elaboration of chemotactic activity for neutrophils and macrophages. These factors cannot be found in lysates of uninfected cells. They do not appear to be associated with the viral particles per se, but rather are present in virus-free supernates from infected fluids. Ultracentrifugal studies of the neutrophil chemotactic activity in allantoic fluid of embryos infected with the two different viruses indicate a similar biphasic distribution of activity, while fluid from the mammalian cell cultures shows a single zone of leukotactic activity, further suggesting that the infected cell, rather than the virus, is responsible for the leukotactic activity.

Virus-infected cells also release a substance(s) which is itself not leukotactic but which can interact with human C3 or C5 to generate such activity. This leukotactic factor–generating substance is similar to that reported in another virus-infected cell system.

It is postulated that the leukotactic factors elaborated as a result of virus infection of cells may play a protective role in vivo.

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