References and Notes

1. H. N. Claman, E. A. Chaperon, R. F. Triplett, *J. Immunol.* **97**, 828 (1966).
2. J. F. A. P. Miller and G. F. Mitchell, *J. Exp. Med.* **128**, 801 (1968).
3. A. J. S. Davies, E. Leuchars, V. Wallis, P. C. Koller, *Transplantation* **4**, 438 (1966).
4. A. J. S. Davies, E. Leuchars, V. Wallis, R. Marchant, E. V. Elliot, *ibid.* **5**, 222 (1967).
5. J. Monod, in *Cellular and Humoral Aspects of the Hypersensitive States*, H. S. Lawrence, Ed. (Hoeber-Harper, New York, 1959), pp. 628–645; N. A. Mitchison, *Symp. Quant. Biol.* **32**, 431 (1967); A. M. Cross and O. Mäkelä, *Immunology* **15**, 389 (1968).
6. D. Naor and D. Sulitzeanu, *Nature* **214**, 687 (1967); *Israel J. Med. Sci.* **5**, 217 (1969).
7. N. K. Jerne and A. A. Nordin, *Science* **140**, 405 (1963); D. A. Rowley and F. W. Fitch, *J. Exp. Med.* **121**, 671 (1965).
8. J. F. A. P. Miller, P. M. deBurgh, G. A. Grant, *Nature* **208**, 1332 (1965).
9. E. E. Sercarz and F. Modabber, *Science* **159**, 884 (1968).
10. B. Rotman, *Proc. Nat. Acad. Sci. U.S.* **47**, 1981 (1961).
11. P. Byrt and G. L. Ada, *Immunology* **17**, 503 (1969).
12. D. DeLuca, J. Decker, E. E. Sercarz, *Fed. Proc.* **29**, 697 (abstr.) (1970).
13. G. L. Ada and P. Byrt, *Nature* **222**, 1292 (1969).
14. F. Modabber and A. H. Coons, *Fed. Proc.* **29**, 697 (abstr.) (1970).
15. J. F. A. P. Miller, *Proc. Roy. Soc. London B* **156**, 415 (1962); R. A. Good, A. P. Dalmasso, C. Martinez, O. K. Archer, J. C. Pierce, B. W. Papermaster, *J. Exp. Med.* **116**, 773 (1962); B. H. Waksman, B. G. Arnason, B. D. Jankovič, *ibid.*, p. 187.
16. K. E. Fichtelius, G. Laurell, L. Philipsson, *Acta Pathol. Microbiol. Scand.* **51**, 81 (1961); L. D. MacLean, S. J. Zak, R. L. Varco, and R. A. Good, *Transplant. Bull.* **4**, 21 (1957).
17. R. B. Taylor, *Nature* **208**, 1334 (1965); D. Metcalf, *ibid.*, p. 1336.
18. D. Metcalf, *Brit. J. Haematol.* **6**, 324 (1960); D. Metcalf and M. Brumby, *J. Cell Physiol.* **67**, 149 (1966).
19. J. F. A. P. Miller, S. M. A. Doak, A. M. Cross, *Proc. Soc. Exp. Biol. Med.* **112**, 785 (1963).
20. R. B. Taylor, *Immunology* **7**, 595 (1964).

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Inflammation and Herpes Simplex Virus: Release of a Chemotaxis-Generating Factor from Infected Cells

Abstract. *Infection of primary rabbit kidney cells with herpes simplex virus leads to the release of a cell factor or factors that upon incubation with serum results in the cleavage of the fifth component, C5, of complement. The product of this cleavage, C5a, is chemotactic for polymorphonuclear leukocytes and could be responsible for the accumulation of these cells at the site of herpetic lesions.*

Although inflammatory cells are often seen at the site of virus-induced tissue destruction (*1*), little is known about the factors responsible for the local accumulation of these cells. It has been postulated that intracellular products released from damaged parenchymal cells might serve as stimulants for inflammation (*2*), but it has been difficult to isolate and characterize these products from in vivo infections. An in vitro system, however, would make it possible to dissect the inflammatory response and isolate the factors released from virus-infected cells. In addition, the ability of these factors to interact with serum components and to specifically attract inflammatory cells could be determined quantitatively by the leukocyte migration technique (*3*). We now describe the release in vitro of a polymorphonuclear leukocyte chemotaxis-generating factor (or factors) from cultures of primary rabbit kidney cells infected with herpes simplex virus and the interaction of this factor with serum complement.

Monolayers of primary rabbit kidney cells (PRK) and stock pools of herpes simplex virus (HSV, type 1) were prepared as described (*4*). Gey's medium or Gey's medium mixed with an equal volume of Eagle's medium (*5*) were used as the diluents. To study the factors released from virus-infected cells, PRK monolayers were infected with HSV diluted in the Eagle-Gey medium. After 3 hours, the inoculum was aspirated; the monolayers were then washed three times and incubated with a fresh portion of the Eagle-Gey medium at 37°C in humidified atmosphere containing 5 percent CO_2. At various times the used medium was removed from the cells and centrifuged at 2000*g* for 10 minutes; the supernatants were separated and stored at −70°C. To test for chemotactic activity, supernatants were incubated alone or with rabbit or guinea pig serum (37°C for 60 minutes) (*6*, *7*). These mixtures then were assayed for chemotactic activity by measuring migration of polymorphonuclear leukocytes (PMN's) through a micropore filter (1.2 μm) by a modification of the Boyden technique (*8*).

To see whether uninfected PRK cells contained factors capable of initiating a chemotactic response, approximately 3×10^7 cell/ml in Gey's medium were sonicated for 2 minutes (4°C) and centrifuged at 10,000*g* for 30 minutes (4°C); the supernatants (uninfected cell lysates) were then tested for chemotactic activity. Table 1 shows that lysates alone had no significant chemotactic activity. Incubation of lysates with homologous rabbit serum, however, resulted in a marked increase in activity. Only a slight increase in chemotactic activity occurred when the lysates were incubated with serum that had been heated at 56°C for 30 minutes. Addition of ethylenediaminetetraacetic acid to the incubation mixture inhibited generation of chemotactic activity, whereas soybean trypsin inhibitor had no significant effect. These experiments show that a chemotaxis-generating factor (CGF) is present in lysates of uninfected PRK cells (*9*).

To see whether HSV infection of PRK cells would result in the release of CGF, PRK monolayers (2×10^6 cells) were infected with 4×10^6 plaque-forming units (PFU) of HSV. At various times thereafter supernatants were removed and tested for CGF activity. Uninfected monolayers served as controls. Release of CGF occurred within 12 hours after infection (Fig. 1) and was associated with the appearance of cytopathology. At the time of maximum release from the infected monolayers, neither release nor cytopathology was detected in uninfected monolayers. The conditions required for the produc-

Table 1. Chemotactic activity generated in rabbit serum by uninfected cell lysate and infected cell supernatant. One milliliter of uninfected cell lysate or infected cell supernatant (collected 22 to 24 hours after infection) was incubated at 37°C for 60 minutes with 1.0 ml of the appropriate reagent, and 0.5 ml of each reaction mixture was assayed for chemotactic activity. PMN, polymorphonuclear leukocyte; EDTA, ethylenediaminetetraacetic acid.

Reagents added	Chemotactic activity (PMN's per high-power field)	
	Uninfected cell lysate	Infected cell supernatant
None	11	8
Serum	185	168
Heated (56°C; 30 minutes) serum	28	40
0.012*M* EDTA (*p*H 7.2) plus serum	32	41
Soybean trypsin inhibitor (5 mg/ml) plus serum	210	144
Serum alone without lysate or supernatant	14	17

tion of chemotactic activity by the infected cell supernatants were identical to those described for uninfected cell lysates (Table 1).

To characterize the chemotactic activity produced by the interaction of CGF with rabbit serum, uninfected cell lysate was incubated with rabbit serum, and the reaction mixture was chromatographed on Sephadex G-75. The resulting chemotactic activity (Fig. 2A) eluted in a single peak immediately prior to the cytochrome c marker (molecular weight, 12,500). Supernatants from infected cells were incubated with rabbit serum and chromatographed in a similar manner. The chemotactic activity of this reaction mixture also eluted in a single peak immediately prior to the cytochrome c marker (Fig. 2B). When guinea pig serum was substituted for homologous rabbit serum the elution profiles of the chemotactic activity were very similar to those seen in Fig. 2, A and B. Sometimes the elution profiles showed a small amount of high molecular weight (greater than 68,000) chemotactic activity; but unlike the low molecular weight material (Fig. 2), this high molecular weight activity was frequently found in normal serum (*7*).

With guinea pig complement, activation of C5 leads to the release of a low molecular weight polypeptide, C5a, which has both anaphylatoxic and PMN chemotactic activities (*10, 11*). On Sephadex filtration (*7*) C5a eluted in a position similar to the chemotactic activity seen in Fig. 2. The next experiment was designed to see whether the chemotactic activity generated by CGF was due to C5a. The peak of chemotactic activity generated by the interaction of CGF and guinea pig serum was isolated by Sephadex filtration and incubated with rabbit antiserum against highly purified guinea pig C3, C5 (*12*), and C5a (*13*). Antiserum to C5 and antiserum to C5a inhibited PMN chemotactic activity whether generated by uninfected cell lysate or by infected cell supernatant (Table 2). Neither antiserum to C3, nor phosphate-buffered saline, nor serum from normal rabbits reduced PMN chemotactic activity.

On the basis of these experiments in vitro we propose the following hypothesis to explain the accumulation in vivo of PMN's at the site of herpetic inflammatory lesions. Infection with HSV, a lytic virus, produces cell damage which results in the release of CGF. At the site of release, CGF interacts with serum complement and leads to the cleavage of C5. A product of this cleavage, C5a, results in the migration of PMN's to the area of virus-induced cell damage.

Although little is known of the nature of CGF, we have evidence that it can directly generate chemotactic activity from highly purified guinea pig C5 (*12*). In this respect CGF is similar to lysosomal enzymes that have been reported to cleave purified C3 and C5 into split products which have chemotactic activity (*14*). Furthermore, lytic viruses are known to release lysosomal enzymes from infected cells (*15*). Thus CGF may be a lysosomal enzyme. It should be emphasized, however, that C5a also can be released from C5 as a result of the activation of the complement sequence by antigen-antibody complexes (*10, 16*). It is possible that "natural antibodies" in rabbit serum may react with intracellular antigens (*17*) in the preparations containing CGF and result in the cleavage of C5. Thus, C5a may be released by either direct enzymatic cleavage of C5 or by the activation of C5 by the earlier acting complement components (*18*).

Humoral and cellular immune responses to virus infections also might play a role in the local accumulation of PMN's. The interaction of antiserum

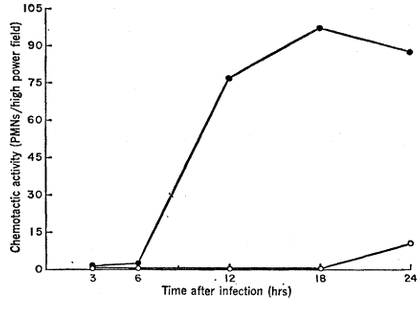

Fig. 1. Release of chemotaxis-generating factor from cells infected with HSV. Monolayers of PRK cells were infected with 4×10^6 PFU of HSV (-●-●-). After 3 hours the inoculum was aspirated, the monolayers were washed three times, and 2 ml of fresh Eagle-Gey medium was added to each plate. Uninfected monolayers served as controls (-○-○-). At various times supernatants were removed, incubated with serum, and tested for chemotactic activity. In the absence of serum, the supernatants had no chemotactic activity. The data are expressed as the chemotactic activity resulting from the interaction of the supernatants with serum minus the background activity of serum alone.

Table 2. Inhibition of chemotactic activity by antiserum to C5 and antiserum to C5a. The reaction mixtures were chromatographed on Sephadex G-75, and the peaks of chemotactic activity were isolated. The isolated material (0.8 ml) was incubated for 15 minutes at 37°C with 0.025 ml of the appropriate antiserum and then tested for residual chemotactic activity. Normal rabbit serum and the rabbit antiserums were inactivated at 56°C for 30 minutes before use. Antiserum to C3 (anti-C3) contained 500 μg of antibody nitrogen per milliliter. Antiserum to C5 contained 120 μg of antibody nitrogen per milliliter. The data for chemotactic activity are corrected for the low levels of chemotactic activity present in some of the rabbit antiserums.

Reaction mixture	Chemotactic activity (PMN's per high-power field)				
	Phosphate-buffered saline	Normal serum	Anti-C3	Anti-C5	Anti-C5a
Uninfected cell lysate plus guinea pig serum	175	210	216	7	0
Infected cell supernatant plus guinea pig serum	146	132	216	17	5

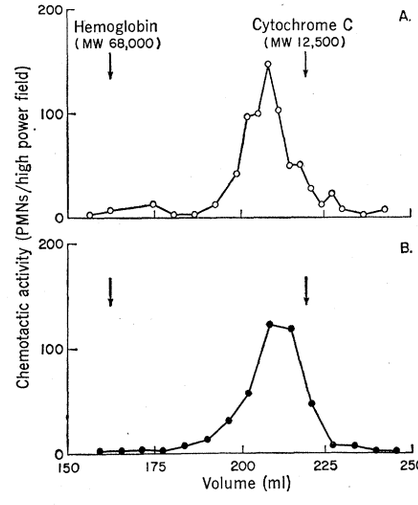

Fig. 2. Fractionation of chemotactic activity by molecular sieve chromatography. (A) Uninfected cell lysate (2.0 ml) was incubated with rabbit serum (4.0 ml) at 37°C for 60 minutes (*21*). The reaction mixture was chromatographed on Sephadex G-75 column (100 by 1.5 cm) and eluted with phosphate-buffered isotonic saline (0.02*M* at *p*H 7.2). Cytochrome c and native human hemoglobin were used as molecular weight markers. Fractions of 3.1 ml were collected, and 1.0 ml was assayed for chemotactic activity. (B) Infected cell supernatant (2.5 ml) collected 24 hours after infection was incubated with rabbit serum (5.0 ml) at 37°C for 60 minutes (*21*), and the reaction mixture was chromatographed as in (A).

to HSV with HSV can activate the complement sequence (*19*) and may lead to the release of C5a. In addition, the interaction of antibody or sensitized lymphocytes with virus-induced antigens on the membrane of infected cells might lead to cell lysis, release of CGF, cleavage of C5, and attraction of PMN's. This might explain the observation of Oldstone and Dixon (*20*) that PMN's accumulated in the leptomeninges of mice infected with lymphocytic choriomeningitis virus after intrathecal administration of antiviral antibody.

The release of intracellular factors from virus-infected cells might be responsible for other aspects of the inflammatory response, such as monocyte and lymphocyte accumulation and increased vascular permeability. The nature and amount of the factors released may depend upon the specific cell type infected as well as on the characteristics of the virus-induced cytopathology. By use of in vitro systems such as described here, it should be possible to characterize some of these factors and further elucidate the mechanism of inflammation in virus infections.

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References and Notes

1. H. Pinkerton, in *Pathology*, W. A. D. Anderson, Ed. (Mosby, St. Louis, 1966), p. 291; E. Friedrich, W. Cole, J. Middelkamp, *Amer. J. Obstet. Gynecol.* **104**, 758 (1969); M. Hogan, S. Kimura, P. Thygeson, *Amer. J. Ophthalmol.* **57**, 551 (1964).
2. F. Sanders, in *General Pathology*, H. Florey, Ed. (Saunders, Philadelphia, 1962), p. 776.
3. S. Boyden, *J. Exp. Med.* **115**, 453 (1962).
4. B. Hampar, A. Notkins, M. Mage, M. Keehn, *J. Immunol.* **100**, 586 (1968).
5. Gey's balanced salt solution (*p*H 7.2) contains 2 percent bovine serum albumin, 62 μg of penicillin, and 135 μg of streptomycin per milliliter. Eagle's minimal essential medium contains 75 μg of neomycin per milliliter.
6. To abolish the low levels of heat-labile chemotactic activity in normal serum (*7*), after the initial incubation at 37°C for 60 minutes all samples were heated for 30 minutes at 56°C prior to testing for chemotactic activity.
7. R. Snyderman, H. Shin, J. Phillips, H. Gewurz, S. Mergenhagen, *J. Immunol.* **103**, 413 (1969).
8. T. Tempel, R. Snyderman, H. Jordan, S. Mergenhagen, *J. Periodontol. Periodontics* **41**, 71 (1970).
9. Preliminary experiments indicate that lysates of cultured HEP-2 cells and of normal rabbit corneas can generate chemotactic activity from normal rabbit serum.
10. H. Shin, R. Snyderman, E. Friedman, A. Mellors, M. Mayer, *Science* **162**, 361 (1968).
11. J. Jensen, R. Snyderman, S. Mergenhagen, in *Cellular and Humoral Mechanisms in Anaphylaxis and Allergy*, H. Movat, Ed. (Karger, Basel, 1969), pp. 265–278.
12. Provided by Dr. H. S. Shin, Department of Microbiology, Johns Hopkins University, Baltimore, Md.
13. Provided by Dr. J. Jensen, Department of Microbiology, University of Miami, Miami, Fla.
14. J. Hill and P. Ward, *J. Exp. Med.* **130**, 505 (1969); P. Ward and J. Hill, *J. Immunol.* **104**, 535 (1970); S. Taubman, P. Goldschmidt, I. Lepow, *Fed. Proc.* **29**, 434 (1970).
15. A. Allison and B. Sandelin, *J. Exp. Med.* **117**, 879 (1963); J. Flanagan, *J. Bacteriol.* **91**, 789 (1966); K. Blackman and H. Bubel, *J. Virol.* **4**, 203 (1969).
16. R. Snyderman, J. Phillips, S. Mergenhagen, *Infect. Imm.* **1**, 521 (1970).
17. J. Kidd and W. Friedewald, *J. Exp. Med.* **76**, 543 (1942).
18. The inhibitory effect of EDTA (Table 1) does not further define the means by which CGF causes cleavage of C5, since the action of a number of proteolytic enzymes, as well as the sequential activation of complement, requires divalent cations.
19. C. Daniels, T. Borsos, H. Rapp, R. Snyderman, A. Notkins, *Proc. Nat. Acad. Sci. U.S.* **65**, 528 (1970).
20. M. Oldstone and F. Dixon, *J. Exp. Med.* **131**, 1 (1970).
21. After incubation at 37°C for 60 minutes these mixtures were heated at 56°C for 30 minutes to inactivate the remaining complement and thus prevent activation of complement during passage through Sephadex G-75.
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Tolerance to Morphine-Induced Increases in [^{14}C]Catecholamine Synthesis in Mouse Brain

Abstract. *Morphine sulfate increased the incorporation of carbon-14–labeled tyrosine into labeled catecholamines in the mouse brain. Tolerance was manifested by a shift to the right in the dose-response curve for morphine after mice were treated repeatedly with morphine. Naloxone, a specific morphine antagonist, also shifted the dose-response curve for morphine to the right.*

Repeated administration of morphine to mice results in the development of tolerance to such specific effects of morphine as analgesia, increased locomotor activity, and decreased brain catecholamine content (*1–3*). These effects of morphine are prevented by specific antagonists (*2–4*). In contrast, morphine has other effects, such as convulsions and tremors, which are not prevented by specific antagonists. Tolerance does not develop to the convulsant actions of morphine (*5*). Morphine (60 mg/kg, injected intraperitoneally) increases the incorporation of [^{14}C]tyrosine into [^{14}C]catecholamines in the rat brain (*6*). We determined the effects of morphine on the incorporation of [^{14}C]tyrosine into [^{14}C]catecholamines in the mouse brain under conditions previously found to produce tolerance to other specific actions of morphine. To further evaluate the effect of morphine on catecholamine synthesis, naloxone, a morphine antagonist, was studied.

Table 1. Antagonism by naloxone (1 mg/kg) of the effects of morphine on incorporation of [^{14}C]tyrosine into [^{14}C]catecholamines in mouse brain. Each value represents the mean ± S.E. of at least five determinations. The *P* values refer to the significance of differences between each pair of values.

Drug dose (mg/kg)	[^{14}C]Catecholamines (dpm/g)	
	Control	Naloxone treated
	Saline	
Control	1817 ± 139	1787 ± 56 ($P > .4$)
	Morphine	
10	2703 ± 198	2214 ± 103 ($P < .05$)
30	3067 ± 320	2439 ± 103 ($P < .05$)
100	2844 ± 210	2671 ± 124 ($P > .2$)
300	2630 ± 176	2521 ± 250 ($P > .4$)

The incorporation of [^{14}C]tyrosine into [^{14}C]catecholamines in brain was determined at various times after the injection of [^{14}C]tyrosine (55 μg/kg; specific activity, 513 mc/mmole, uniformly labeled, Amersham/Searle) into the tail vein of the female Swiss-Webster mouse (20 to 30 g). Brain [^{14}C]catecholamines were isolated on alumina as previously described (*7*).

The maximum incorporation into [^{14}C]catecholamines occurred 40 minutes after the injection of [^{14}C]tyrosine (Fig. 1). A marked increase in incorporation into [^{14}C]catecholamines was observed when the [^{14}C]tyrosine was injected 30 minutes after the injection of morphine sulfate (100 mg/kg, intraperitoneally). After this dose of morphine, the maximum incorporation also occurred 40 minutes after the injection of [^{14}C]tyrosine (Fig. 1). Therefore, dose-response relations for morphine sulfate were determined 70 minutes after morphine administration and 40 minutes after injection of [^{14}C]tyrosine. Morphine (10 to 300 mg/kg, injected intraperitoneally) increased incorporation of [^{14}C]tyrosine into [^{14}C]catecholamines. The maximum increase occurred at a dose of 30 mg/kg (Fig. 2, solid circles). The increased incorporation of [^{14}C]tyrosine into [^{14}C]catecholamines did not result from a morphine-induced increase in the specific activity of [^{14}C]tyrosine in the mouse brain. Morphine (100 mg/