1-1-1979

Cytokine production by blue tongue virus-infected fetal sheep cells

F. M. Enright
University of California, Davis

B. I. Osburn
University of California, Davis

Recommended Citation

Enright, F., & Osburn, B. (1979). Cytokine production by blue tongue virus-infected fetal sheep cells. *Infection and Immunity, 25* (1), 403-407. https://doi.org/10.1128/iai.25.1.403-407.1979

INFECTION AND IMMUNITY, July 1979, p. 403-407
0019-9567/79/07-0403/05$02.00/0

Vol. 25, No. 1

Cytokine Production by Blue Tongue Virus-Infected Fetal Sheep Cells

F. M. ENRIGHT†* AND B. I. OSBURN

Department of Veterinary Pathology, University of California, Davis, California 95616

Received for publication 6 April 1979

The migration inhibition of guinea pig peritoneal macrophages by a factor(s) from media obtained from blue tongue virus-infected monolayer cultures was studied. Medium from blue tongue virus-infected sheep fetal cell cultures inhibited migration of guinea pig macrophages from agarose droplets. Medium from control cultures and stock virus did not inhibit macrophage migration. Medium containing migration inhibiting factor(s) in vitro induced an inflammatory reaction in the skin of a newborn sheep. The inflammatory reaction was observed 20 h after intradermal inoculation. The skin reaction consisted of infiltrates of mononuclear leukocytes in the superficial dermis. Control medium and stock virus caused no skin reaction.

An inflammatory response characterized by infiltration of macrophages is a prominent extraneural lesion in sheep fetuses infected with blue tongue virus (BTV). At 10 to 15 days postinfection, diffuse infiltrates of large mononuclear leukocytes are present in the interstitial tissue of the lung and other parenchymatous organs. This diffuse reaction was replaced by a focal unitized granulomatous reaction in fetuses infected 20 days or longer (F. M. Enright, Ph.D. thesis, University of California, Davis, Calif., 1974). The character and intensity of the inflammation was unchanged as fetal animals developed specific humoral immune responses to the virus. These reactions are similar to reactions in other congenital infections caused by viruses, bacteria, and protozoa. This type of cellular inflammatory response may represent the earliest and most primitive fetal defense mechanism (6, 7).

Infection of different nonlymphoid cell lines with various viruses produces a migration inhibition (MIF)-like factor. This factor is chemically similar to MIF produced by lymphocytes (2). Such factors are referred to as "cytokines" to differentiate them from the biologically active factors produced by sensitized lymphocytes (1, 3, 4, 8, 11-13). It was felt that cytokine production by virus-infected cells may account for the inflammatory lesions observed in infected fetuses.

In this study, cell cultures from fetal lamb brain, lung, and kidney were infected with BTV. Supernatants from infected cell cultures were examined for MIF activity and skin-reactive factors. The significance of these biologically active factors in the production of the fetal inflammatory response is discussed.

† Present address: Department of Veterinary Pathology, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803.

MATERIALS AND METHODS

Cell cultures. Monolayer cell cultures of cerebral cortex, lung, and kidney were established from explants of normal fetal sheep brain, lung, and kidney. These tissues were removed from control fetuses on days 50, 65, 100, and 130 of gestation. Cell cultures were grown in 75-cm^2 flasks. Growth medium consisted of Eagle minimal essential medium with Earle balanced salt solution and 10% heat-inactivated, virus-screened fetal bovine serum and antibiotics. Cultures were incubated at 37°C in a humidified CO_2 atmosphere. Cultures in this study were used between their second and eighth passages.

Virus preparation. Serotype 10 of BTV was used in these studies. Stock virus suspensions were prepared from tissue culture media collected from infected sheep fetal kidney cell cultures. Cultures were infected with BTV, and the media were removed 4 to 5 days later when cytopathic effects were estimated to be 80 to 100%. This media was subjected to low-speed centrifugation (2,000 × *g*) to remove cell debris. The supernatants were collected and placed in sterile vials and maintained for up to 1 month at 4°C. Infectivity assays were performed by inoculating 0.025 ml of 10-fold dilutions of the virus stock suspension into microtiter cultures of sheep fetal kidney cells, observing the cultures for 5 days for cytopathic effect, and then calculating the virus titer by the method of Reed and Muench (9). The stock virus titers were found to be 5 × 10^5 to 5 × 10^6 50% tissue culture infective doses per 0.025 ml of stock virus.

Production of cytokine. After passage, the various fetal cell cultures were incubated in a CO_2 atmosphere at 37°C until confluency of the monolayers was observed. During this period of incubation, generally

2 to 4 days, 5 ml of tissue culture media was removed and centrifuged at 800 × *g* at 4°C, and the supernatants were stored at −10°C until tested for MIF-like activity.

When cell monolayers had become confluent, medium was removed and the cell sheets were washed twice with 20 ml of phosphate-buffered saline. A 25-ml volume of fresh complete media was then added to the cultures. At 24 h after the addition of fresh medium, designated cultures were infected with stock BTV. One-milliliter volumes of media from infected and control cultures were removed 24, 30, 36, and 48 h postinfection. These samples were treated as above. After 48 h, the volume of media in each culture was brought up to 25 ml per flask, and the cultures were maintained until cytopathic effects were observed, generally on day 4 or 5 postinfection.

Migration inhibition tests. Macrophage migration inhibition from a droplet of agarose was used to determine the presence of migration inhibition factors in supernatants of fetal cell cultures (5). Adult guinea pigs were inoculated intraperitoneally with 25 ml of light mineral oil. At 4 to 5 days later, the guinea pigs were killed and the peritoneal cavity was washed with 50 ml of minimal essential medium. Peritoneal fluids were collected, and the nonlipid-containing layer of media and cells was transferred to a conical tube and centrifuged at 300 × *g* for 10 min at room temperature. The cell pellet was suspended in fresh minimal essential medium and recentrifuged. This cell washing was repeated three times, and the final washed pellet was suspended in 10 ml of minimal essential medium. Droplets of the cell suspension were placed on microscope slides and stained with trypan blue to determine cell viability. Viability greater than 70% was required for these studies. Differential cell counts were made on smear preparations of the suspension on glass cover slips and stained with Wright's stain. Cells were not used except when greater than 50% of the cells had the morphological appearance of macrophages. These macrophages contained little phagocytized lipid. The 10-ml cell suspension was centrifuged as described, and the supernatant was discarded. The cell pellet was mixed with an equal volume of 0.4% agarose in minimal essential medium at 40°C. A 1-μl pipette was used to dispense the agarose cell mixture in wells of a flat-bottom microtiter plate. Plates were placed in a humidified chamber and refrigerated at 4°C for 15 min. After refrigeration, 0.1 ml of the various supernatants to be tested was carefully added to each well. The plates were incubated in a humidified chamber in a 5% CO_2 atmosphere at 37°C. At 1 to 5 h after preparing the test, initial measurements of each droplet were made by use of a dissecting microscope with a micrometer eyepiece. Measurements were made at a total magnification of ×30, with the diameters of the droplets determined along horizontal and vertical lines. Measurements were repeated 24 and 48 h after the initial measurement. After the final measurements were made, the cells in selected wells which contained inhibited and noninhibited macrophages were stained with trypan blue. Viability of 60% or more was required for each test supernatant. The average migration of cells from these droplets was recorded, and the percent migration inhibition was determined by the formula: percent inhibition = 1 − [(mean units of migration in test supernatants)/(mean units of migration in control supernatants)] × 100. Control supernatants in computation of the percent inhibition was based on migration of macrophages in fresh complete media. Other controls for tests were the virus stock suspension and medium removed from age-matched noninfected cultures. Migration inhibition of greater than 20% was considered significant.

Skin tests. Various supernatants from infected and noninfected fetal cell cultures were tested for in vivo inflammatory properties by inoculating 0.1 ml or less of the test material intradermally into newborn lambs. This material was tested for sterility on tryptose broth. Tissue culture media, virus stock suspensions, and media from uninoculated cell cultures were included as controls. The inoculation sites were observed 20 h after inoculation for gross alterations, biopsied, fixed immediately in 10% buffered Formalin, and processed by routine histological techniques.

RESULTS

In vitro studies. No significant inhibition of macrophage migration was noted in media controls, virus controls, or in cell culture controls once confluency of the monolayer was established (Fig. 1). Media removed from cell controls during the interval of cell growth prior to becoming a confluent monolayer demonstrated up to 30% inhibition of migration. In several instances, media removed from noninfected cerebral cortex cultures from 65- and 130-day-old fetuses demonstrated slight increases in the percent inhibition of migration. In both instances, confluency of the monolayers was disrupted by the saline washings.

The media from virus-infected monolayers contained substances which inhibited the migration of macrophages by 40% or more (Fig. 2). Macrophage migration was most inhibited 24 to 36 h after initiating the tests. By 72 h, migration inhibition was no longer significant.

In all instances a MIF-like activity was observed in infected cultures. When cytopathic effect was estimated to be 80 to 100% the MIF-like activity stabilized at 30 to 40% inhibition of migration.

The substance(s) responsible for the inhibition of migration activity observed in the culture media was nondialyzable and heat stable at 56°C for 30 min. BTV and the various factors in the supernatants from virus-infected cells did not demonstrate any toxic effects in macrophages. Cell viability based on trypan blue exclusion remained unaltered, and the macrophages initially inhibited by the factor resumed normal migration ability by 48 h and was completed by 72 h after initiation of the MIF assay.

In vivo studies. By 20 h, skin sites where MIF-containing supernatants had been inoculated were slightly indurated. The induration was accompanied by a cellular inflammatory

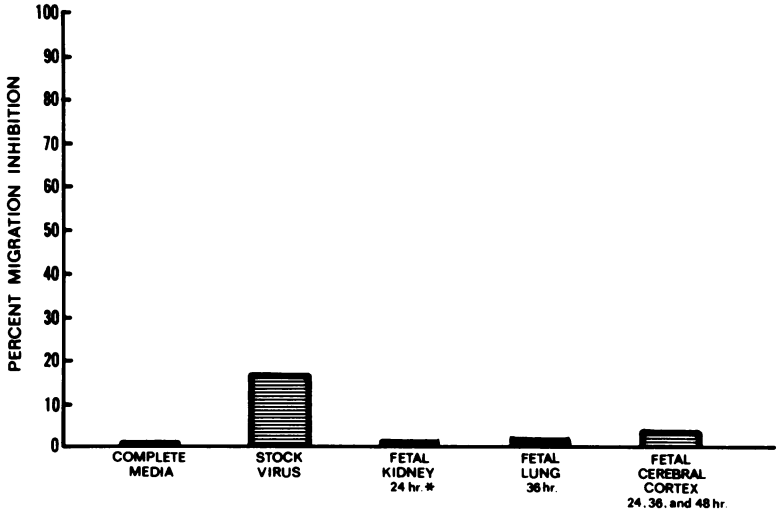

FIG. 1. *Migration inhibition activity of supernatants from uninfected control fetal sheep cultures. Asterisk indicates age-matched control cultures with reference to the time the principal cultures had been infected with BTV.*

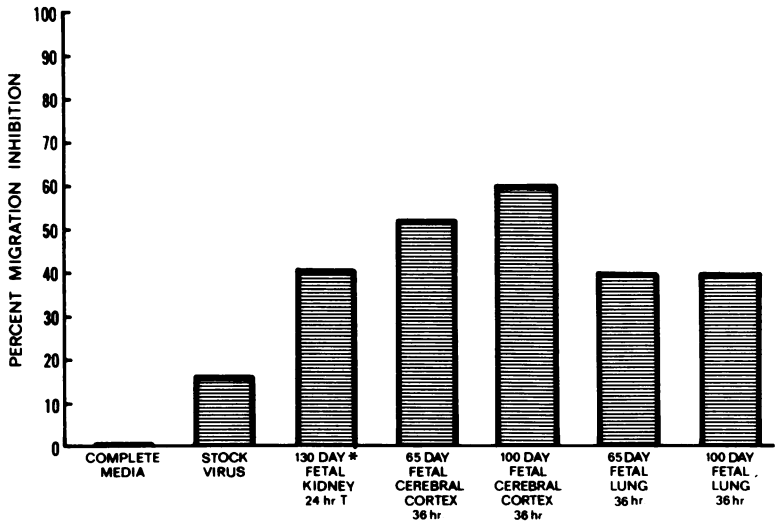

FIG. 2. *Migration inhibition activity of supernatants from BTV-infected fetal sheep cultures. Asterisk indicates age of fetus when tissue was explanted. T, Time interval after virus infection (hours).*

reaction composed of mononuclear leukocytes and a few polymorphonuclear leukocytes. These inflammatory cells were within venules and in connective tissue adjacent to blood vessels (Fig. 3 and 4). Inoculation with complete tissue culture media, virus stock, and supernatants from noninfected cultures did not elicit an inflammatory response.

DISCUSSION

BTV infection of fetal sheep cell cultures produces a factor or factors which inhibit the migration of macrophages in vitro. The biological activity and some observations of its physical properties indicate that it is similar to the factors produced by cells infected with mumps virus, Newcastle disease virus, and simian virus (1, 3, 8, 11–13). Low levels of MIF activity in nonconfluent monolayers were expected, as it is produced during the growth phases prior to establishing a confluent monolayer (10).

The inflammatory reaction observed in the skin of newborn lambs, inoculated with supernatants demonstrating in vitro migration inhibition activity, suggests that several factors, including a chemotactic factor to mononuclear

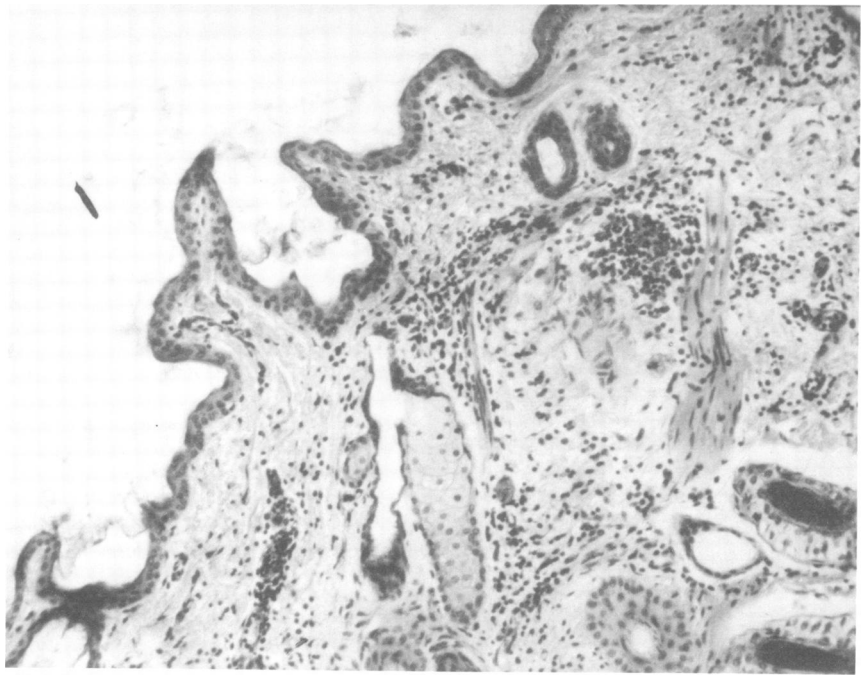

FIG. 3. *Inflammatory reaction in the dermis of a newborn lamb 20 h after intradermal inoculation with a test supernatant which inhibited the migration of macrophages in vitro (×100).*

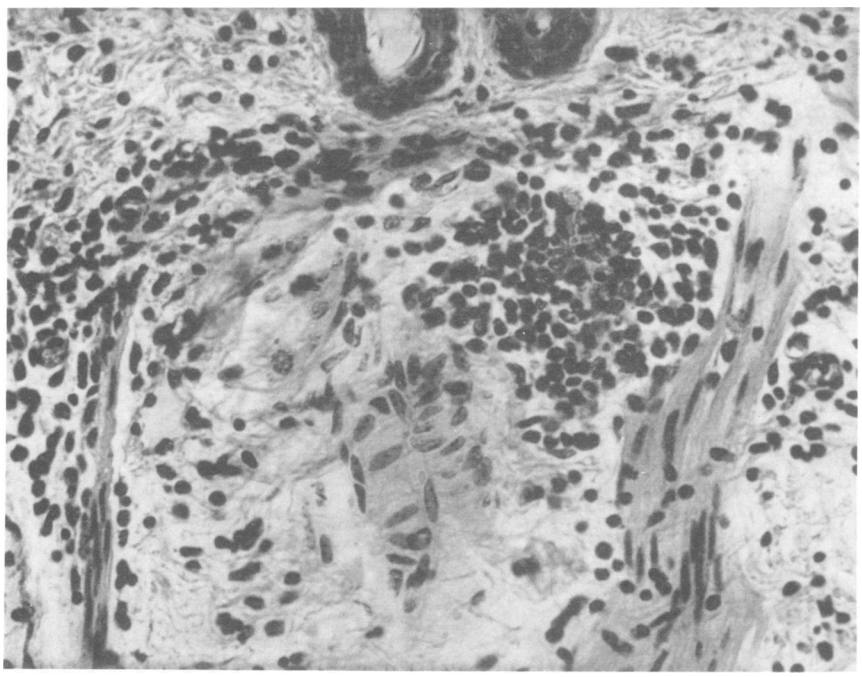

FIG. 4. *Higher magnification of the dermal reaction observed in Fig. 3. Note that a majority of cells are mononuclear and that they are either within or immediately adjacent to small blood vessels (×200).*

leukocytes, may be present in such supernatants. This was also observed in chicken embryos and monkey kidney-derived tissue cultures infected with mumps virus or with Newcastle disease virus (11, 12).

It appears that the mononuclear inflammatory reactions observed in BTV-infected sheep fetuses is one of the most primitive cellular defense mechanisms. Support for this suggestion is borne out by (i) the fact that the mononuclear responses occur in infected fetuses prior to the development of specific immunological responses and (ii) factors capable of generating such forms of inflammation were demonstrated in nonlymphoid fetal cell cultures infected with the virus.

The ability of the virus-infected cells to produce phlogistic factors helps explain why a number of congenital infectious diseases caused by a variety of agents are morphologically similar with respect to the inflammatory reactions observed. Such a mechanism may be responsible for the mononuclear lesions reported with bacterial and chemical irritants in fetal lambs, monkeys, calves, and humans (6, 7).

LITERATURE CITED

1. **Bigazzi, F. E., T. Yoshida, P. A. Ward, and S. Cohen.** 1975. Production of lymphokine-like factors (cytokines) by simian virus 40 infected and simian virus 40 transformed cells. Am. J. Pathol. **80**:69–77.
2. **Cohen, S.** 1977. The role of cell-mediated immunity in the induction of inflammatory responses. Am. J. Pathol. **88**:501–528.
3. **Flanagan, T. D., T. Yoshida, and S. Cohen.** 1973. Production of macrophage migration inhibition factors by virus-infected cell cultures. Infect. Immun. **8**:145–150.
4. **Hammond, M. E., R. O. Roblin, A. M. Dvorak, S. S. Selvaggio, P. H. Black, and H. F. Dorak.** 1974. MIF-like activity in simian virus 40 transformed 3T3 fibroblast cultures. Science **185**:955–957.
5. **Harrington, J. T., Jr., and P. Stastny.** 1973. Macrophage migration from an agarose droplet: development of a micromethod for assay of delayed hypersensitivity. J. Immunol. **110**:752–759.
6. **Morison, J. E.** 1970. Foetal and neonatal pathology, p. 507–525. Butterworths, London.
7. **Osburn, B. I.** 1974. Animal models for the study of immunologic modulation of fetal infectious disease processes, p. 25–32. *In* E. Neter and F. Milgrom (ed.), The immune system and infectious diseases, 4th international convocation on immunology. S. Karger, Basel.
8. **Papageorgiou, P. S., W. L. Henley, and P. R. Glade.** 1972. Production and characterization of migration inhibitory factor(s) (MIF) of established lymphoid and nonlymphoid cell lines. J. Immunol. **108**:494–504.
9. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating 50 percent end points. Am. J. Hyg. **27**:493–497.
10. **Tubergen, D. G., J. D. Feldman, E. M. Pollack, and R. A. Lerner.** 1972. Production of macrophage migration inhibition factor by continuous cell lines. J. Exp. Med. **135**:255–266.
11. **Ward, P. A., S. Cohen, and T. D. Flanagan.** 1972. Leukotactic factors elaborated by virus-infected tissues. J. Exp. Med. **135**:1095–1103.
12. **Yoshida, T., and S. Cohen.** 1974. *In vivo* manifestation of lymphokine and lymphokine-like activity, p. 43–60. *In* R. T. McClustesky and J. Cohen (ed.), Mechanisms of cell-mediated immunity. John Wiley & Sons, New York.
13. **Yoshida, T., T. D. Flanagan, J. Gencor, and S. Cohen.** 1974. Virus-induced migration inhibitory activity in experimental mumps infection. Clin. Immunol. Immunopathol. **2**:472–480.