Ann. N. Y. Acad. Sci., 1954, v58, 613.
3. Field, E. O., J. Neurol. Neurosurg. and Psychiat., 1954, v17, 228.
4. Press, E., Biochem. J., 1956, v63, 367.
5. MacPherson, C. F. C., Cosgrove, J. B. R., Canad. J. Biochem. and Physiol., 1961, v39, 1567.
6. MacPherson, C. F. C., ibid., 1962, v40, 1811.
7. MacPherson, C. F. C., Saffran, M., J. Immunol., 1965, v95, 629.
8. Hochwald, G. M., Thorbecke, G. J., Proc. Soc. Exp. Biol. and Med., 1962, v109, 91.
9. Laterre, E. C., Heremans, J. F., Carbonara, A., Clin. Chim. Acta, 1964, v10, 197.
10. Laterre, E. C., Heremans, J. F., ibid., 1963, v8, 220.
11. Hochwald, G. M., Thorbecke, G. L., ibid., 1963, v8, 678.
12. ———, Arch. Biochem. and Biophys., 1963, v101, 325.
13. Dencker, S. J., Swahn, B., Nature, 1962, v194, 288.
14. Clausen, J., Proc. Soc. Exp. Biol. and Med., 1961, v107, 170.
15. Huntley, C. C., Pediatrics, 1963, v31, 123.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., J. Biol. Chem., 1951, v193, 265.
17. Grubb, R., Laurell, A. B., Acta Path. et Microbiol. Scand., 1956, v39, 390.
18. Harboe, M., ibid., 1959, v47, 191.
19. Martensson, L., J. Exp. Med., 1964, v120, 1169.
20. Ropartz, C., Lenoir, J., Rivat, L., Nature, 1961, v189, 586.
21. Harboe, M., Lundevall, J., Acta Path. et Microbiol. Scand., 1959, v45, 357.
22. Ceppellini, Dray, S., Edelman, G., Fahey, J., Franěk, F., Franklin, E., Goodman, H. C., Grabar, P., Gurvich, A., Heremans, J. F., Isliker, H., Karush, F., Press, E., Trnka, Z., Bull. World Hlth. Org., 1964, v30, 447.
23. Epstein, W. V., Tan, M., Gross, D., Nature, 1964, v202, 1175.
24. Tan, M., Epstein, W., J. Lab. Clin. Med., 1965, v61, 344.
25. MacPherson, C. F. C., Clin. Chim. Acta, 1965, v11, 298.
26. Welch, K., Friedman, V., Brain, 1960, v83, 454.
27. Welch, K., Pollay, M., Am. J. Physiol. 1961, v201, 651.
28. Allen, J. C., Kunkel, H. G., Kabat, E. A., J. Exp. Med., 1964, v119, 453.
29. Steinberg, A. G., Progress in the Study of Genetically Determined Human Gamma Globulin Types (the Gm and Inv Groups), in Progress in Medical Genetics, A. G. Steinberg, A. G. Bearn, eds., Grune & Stratton, 1962.
30. Alter, M., Allison, R. S., Talbert, O. R., Kurland, L. T., World Neurol., 1960, v1, 55.

Received March 3, 1966. P.S.E.B.M., 1966, v122.

Challenge Virus Resistance and Interferon Produced in BS-C-1 Cells By Dengue Virus. (31190)

PHILIP K. RUSSELL, JOSEPH A. BELLANTI, EDWARD L. BUESCHER AND JACK M. MCCOWN (Introduced by L. S. Baron)
Departments of Virus Diseases, Walter Reed Army Institute of Research, and Pediatrics, Georgetown University Medical School (JAB), Washington, D.C.

Dengue viruses infect mammalian cell cultures without producing obvious cytopathic effect(1). Halstead showed that continuous cultures of African green monkey kidney cells (BS-C-1) or rhesus kidney cells (LLC-MK2) infected with dengue viruses resist superinfection with yet another virus(2). This challenge virus resistance (CVR) is superficially similar to that induced by rubella virus in BS-C-1 cells(3) and by tick-borne encephalitis viruses in chick embryo cell cultures(4), and has been useful for the recovery of dengue viruses from patients' blood. This technique is more sensitive and efficient than is the use of the suckling mouse for isolation and characterization of some strains of dengue viruses(5). The present experiments show that dengue virus-induced CVR is accompanied by the elaboration of an interferon-like substance. The characterization of this interferon, its behavior in BS-C-1 cells, and the circumstances of its production are the subject of this report.

Materials and methods. Viruses. The dengue virus (PR-38) was isolated from a patient during the epidemic in Puerto Rico in 1963(5). The strain was recovered originally in BS-C-1 cell monolayers; third and fourth

BS-C-1 passages were used in these experiments. The virus is closely related antigenically to dengue type 3. Polio type 2 virus used for detection of CVR was recovered in 1956 in this laboratory.

Cell cultures. The BS-C-1 line of continuous GMK cells was obtained in approximately the 300th passage* and subcultured serially. Growth medium was M-199 containing 15% inactivated (56°C, 30 min) fetal bovine serum (FBS), penicillin 100 units/ml, and streptomycin 100 μg/ml. Maintenance medium was Eagle's basal medium (BME) containing 2% inactivated chicken serum and the same antibiotics. BS-C-1 cells were maintained in 250 ml bottles and passaged at 4- to 6-day intervals. Tube cultures were inoculated 4 to 5 days after seeding when a confluent monolayer had formed. Tubes were maintained in stationary racks at 36°C. Maintenance medium was changed every third or fourth day to maintain an alkaline pH.

Infectivity titrations. Infective virus was titrated in serial 10-fold decrements; 0.1 ml of each dilution of virus in maintenance media was inoculated into each of 4 to 6 BS-C-1 tube cultures. Cultures were incubated for 9 days; medium was changed when necessary to maintain pH. CVR was measured on the ninth day by addition of approximately 1000 TCD_{50} of polio virus II. Survival of more than 50% of challenged cells at times when complete destruction of the cell sheets occurred in control tubes (usually 48 hours after inoculation of the polio virus) was interpreted as positive challenge virus resistance (CVR). End points were calculated by the method of Reed and Muench(6).

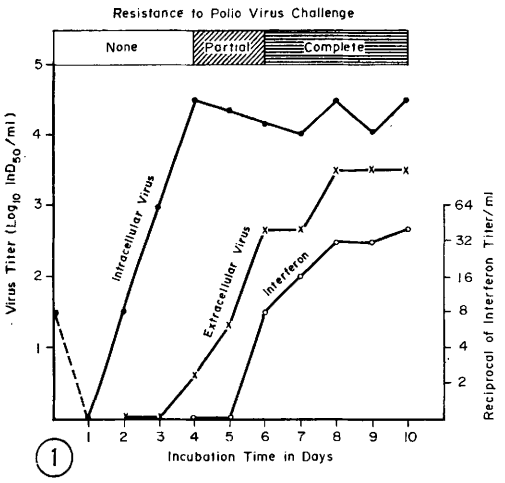
Interferon assay. Infective virus in culture fluids was destroyed by adjusting the pH to 2, with 0.1 N HCl at 4° for 18 hours. The pH of the fluids was returned to 7.2-7.4 with 0.1 N NaOH and serial 2-fold dilutions made in maintenance media. One ml amounts of each dilution were added to BS-C-1 tubes after removal of the media. A minimum of 4 tubes per dilution was used. Tubes were incubated for 24 hours at 36°C, the fluids removed and replaced with maintenance medium containing 1000 ID_{50} of polio virus II. Tubes were observed for CPE and the criteria described above were used to determine the end points of interferon activity.

Gel filtration. Sephadex G-200 in 70 × 2 cm columns was used for filtration experiments. The Sephadex was washed and equilibrated with 0.15 M phosphate buffered normal saline, pH 7.3 (PBS). The column was packed with 10 g of Sephadex under gravity flow. A two ml sample of tissue culture fluid was applied to the column and elutions made with PBS. Two ml fractions were collected, and protein concentrations were determined by the method of Lowry(7). Antibiotics were added to the eluates, and 50% PBS was added to the fractions to be tested for virus content.

Results. Growth of dengue virus within infected cells and release of virus into medium were found to have a consistent relationship to development of CVR and production of an interferon-like substance. Following eclipse of 24 hours or more, virus became detectable in cells; obvious CVR was observed only after maximal concentrations of intracellular virus were attained. Finally, increasing concentrations of interferon-like substances in the medium were demonstrated.

Temporal relationships between virus proliferation, CVR and interferon production. The relationships between virus growth, CVR and the production of interferon were investigated in stationary tube cultures. In each experiment approximately 100 tubes were inoculated with 10 to 3200 interfering doses 50 (InD_{50}) of dengue virus. At 24-hour intervals 10 tubes were selected at random; media and washed cell sheets were harvested separately for determination of infectious virus and interferon content. A sample of pooled cell-free medium for virus titration was mixed with an equal amount of PBS, shell frozen and stored at −70°C. The remaining undiluted medium was frozen and stored similarly. Cells were washed with an excess of BME, and 1 ml of BME containing 50% FBS was added to each tube. Cells were disrupted by agitating with sterile fine glass

* Obtained from Mrs. Hope E. Hopps, Division of Biologics Standards, Nat. Inst. Health.



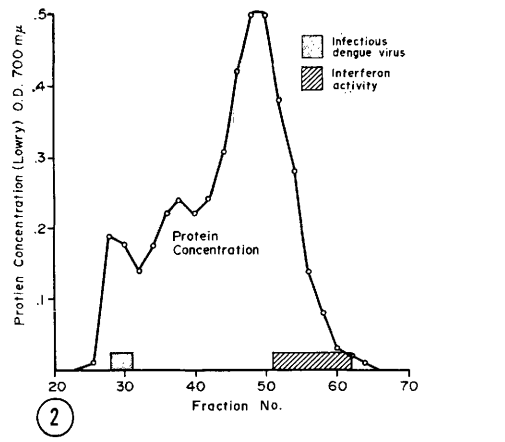


FIG. 1. Virus growth and interferon production in BS-C-1 cells following dengue virus infection.

FIG. 2. Sephadex G-200 separation of dengue virus and interferon.

beads using a Vortex mixer. The resulting suspensions were pooled, shell frozen and stored at —70°C. All materials were thawed and centrifuged at 200 rpm in a refrigerated centrifuge prior to titration. Finally, 4 tube cultures selected randomly from the infected culture stock were challenged with 1000 ID_{50} of polio virus on each day and observed for CVR.

Following inoculation of approximately 32 InD_{50} of dengue virus, infective virus was not detectable in either cells or supernatant media after 24 hours of incubation (Fig. 1). At 48 hours cell associated virus titered $10^{1.5}$ InD_{50}/ml and increased daily reaching peak infectivity of $10^{4.5}$ InD_{50} on the 4th day. Extracellular virus was first detected on the 4th day ($10^{0.7}$/ml), at or near the time when cell associated virus titers were maximal, and increased slowly during the next 4 days. Interferon became detectable on the 6th day (1:8/ml) 48 hours after extracellular virus could be demonstrated, and increased approximately 4-fold during the next 4 days. Partial CVR was observed concurrently with maximal cell associated virus; CVR was not complete, however, until after interferon was detected in the supernatant medium. In another experiment with a small initial virus input, pH of maintenance medium was allowed to become acid. Here, extracellular virus was detected only on the 4th and 5th days, and cell associated virus declined from a peak of 10^4 InD_{50}/ml on the 4th day to 10^2 InD_{50}/ml on day 10. Patterns of CVR were essentially similar to those shown in Fig. 1. With virus inputs of 10^4 InD_{50}, both cell associated and extracellular virus were detected more rapidly; maximal titers of intracellular virus reached $10^{5.5}$ InD_{50}/ml by the 3rd post inoculation day. Appearance of CVR was accelerated, being complete by the 3rd day.

The relationship of the size of the inoculated dose of dengue virus to the appearance of CVR under standard conditions is shown in Table I. With less than 10^2 InD_{50}, appearance of CVR was delayed until as late as the 5th post inoculation day. With greater inputs the delay was correspondingly less. Complete CVR regularly could be shown 1 to 2 days after partial resistance was demonstrated.

Characteristics of dengue-induced BS-C-1 interferon. The interfering substance produced by the dengue-infected BS-C-1 cells

TABLE I. Influenza of Inoculum Size on Appearance of Challenge Virus Resistance in BS-C-1 Cells.

Inoculum (InD_{50})	Occurrence of CVR by indicated days		
	None	Partial	Complete
$10^{0.5}$	0–4	5–6	7–10
10^1	0–4	5	6–10
10^2	0–3	4	5–10
10^3	0–2	3	4–10
10^4	0–1	2	3–10
10^5	0–1	2	3–10

TABLE II. Properties of Interferon Produced by BS-C-1 Cells Following Dengue Virus Infection.

Treatment	Interferon titer Untreated	Treated
Heat (56°C, 1 hr)	1:32	1:2
Heat (60°C, 1 hr)	1:32	<1:1
Trypsin digestion (.5 mg/ml, 37°C, 1 hr)	1:64	<1:1
Ether treatment (20%, 4°C, 18 hr)	1:32	1:8
pH 2 (4°C, 24 hr)	1:64	1:64
pH 1 (4°C, 24 hr)	1:64	<1:1
Ultracentrifugation (100,000 × *g*, 4 hr)	1:64	1:64
Dengue 3 antiserum (4°C, 18 hr)	1:64	1:64

was identified as an interferon by determining its stability at pH 2, sensitivity to digestion by trypsin, lack of inhibition by potent antidengue 3 serum, and sedimentation characteristics by ultracentrifugation. These physical and chemical characteristics are presented in Table II. It meets the criteria established by Isaacs(8) and Wagner(9) for interferon. Heating to 56°C for 1 hour destroyed over 90% of the interferon activity and 60°C for 1 hour resulted in complete loss of activity. This heat lability is similar to that reported for some mouse and human interferons(10,11).

Separation of infectious virus and interferon by gel filtration. Sephadex G-200 fractionation of infected tissue culture fluids without concentration was found to be unsatisfactory because the concentration of interferon was too low to assay in eluted fractions. Infectious dengue virus, however, was readily recoverable from the early fractions associated with the high molecular weight substances. Ten-fold concentration of tissue culture fluids by lyophilization and rehydration to 1/10 the original volume increased the interferon titer to 1:512. When the concentrated material was fractionated and undiluted alternate fractions tested for infectious virus and acid-stable interfering substance, a definite separation of the infectious virus from the interferon activity was obtained (Fig. 2). The infectivity was again found only in the early fractions (26-32) and the interferon activity was detected only in fractions 51 through 62 in the descending portion of the low molecular weight protein peak. Quantitative recovery of the interferon activity after fractionation could not be made by this method probably because of the adsorption of partially purified interferon onto glass(12).

Discussion. Present studies indicate that dengue-infected BS-C-1 cells produce an interferon that has physical and chemical characteristics similar to those reported for other mammalian interferons. It was found feasible not only to separate dengue virus from the interferon by gel filtration, but to do so without significantly destroying the infectivity of the virus. Infectious virus was eluted with those proteins whose molecular size was large enough to prevent them from entering the gel. No acid stable interfering substance was found in those fractions. Interferon activity was eluted with proteins of molecular weight 70,000 or less.

The relationship of the onset of CVR and interferon production makes the hypothesis that the interference is mediated by interferon an attractive one. Interferon is found in the media only after CVR has been established. Presumably, this reflects excess interferon not absorbed by cells. The CVR demonstrated prior to detection of interferon in the media may be due to interferon remaining within infected cells, or to that absorbed to non-infected cells. Alternatively, the biological assay for interferon may be too insensitive to detect its earliest appearance in the media. Time of onset of CVR varies with the infecting dose of dengue virus, and the appearance of interferon in the media varies in the same manner, becoming detectable on the second day after partial CVR is noted, corresponding with the establishment of complete CVR.

Vilcek has shown that CVR afforded chick embryo fibroblasts by infection with tick-borne encephalitis viruses is mediated by a protein indistinguishable from influenza virus induced chick embryo interferon(13). The present experiments suggest that interferons also mediate the resistance of BS-C-1 cells infected with dengue viruses to superinfection with polio virus II. If interferons are indeed responsible for cell resistance to superinfection in this system, host cell-virus relationships must differ significantly from those

of the rubella virus-induced CVR in the same cell, for no interferon-like substances appear to be elaborated by BS-C-1 cells infected with rubella virus(5). No explanation for these differences can currently be made.

Experiments reported elsewhere have confirmed the utility and relative simplicity of the BS-C-1 interference system for the isolation of dengue viruses of several types from human serum, and for performing virus neutralization tests to identify the agents isolated (5). The current experiments define optimal times for harvesting virus and for detecting interfering agents by challenge. Seed viruses for neutralization tests, containing minimal amounts of interferon, can be obtained by harvesting washed cell sheets on the fourth or fifth day of incubation.

Summary. Infection of BS-C-1 cells by dengue virus produces resistance to challenge with polio virus type II. The interference is related to the production of an interferon which is released into the media following the time when maximal intracellular dengue virus titers are present. The interferon appears similar to other mammalian interferons and can be separated from the infectious virus by gel filtration with Sephadex G-200. The use of this method for isolation and identification of dengue viruses is discussed.

1. Hotta, S., Evans, C. A., Proc. Soc. Exp. Biol. and Med., 1956, v93, 53.
2. Halstead, S. B., Sukhavachana, P., Nisalak, A., ibid., 1964, v115, 1062.
3. Parkman, P. D., Buescher, E. L., Artenstein, M. S., McCown, J. M., Mundon, F. K., Druzd, A. D., J. Immunol., 1964, v93, 595.
4. Vilcek, J., Acta Virologica (Prague), 1960, v4, 308.
5. Russell, P. K., Buescher, E. L., McCown, J. M., Ordonez, J., Amer. J. Trop. Med. and Hyg., in press.
6. Reed, L. J., Muench, H., Am. J. Hyg., 1938, v27, 493.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., J. Biol. Chem., 1951, v193, 265.
8. Isaacs, A., Adv. Virus Res., 1963, v10, 1.
9. Wagner, R. R., Ann. Rev. Microbiol., 1963, v17, 285.
10. Merigan, T. C., Science, 1964, v145, 811.
11. Wheelock, E. F., Sibley, W. A., Lancet, 1964, v1, 382.
12. Lampson, G. P., Tytell, A. A., Nemes, M. M., Hilleman, M. R., Proc. Soc. Exp. Biol. and Med., 1963, v112, 468.
13. Vilcek, J., Acta Virologica (Prague), 1962, v6, 144.

Received March 4, 1966. P.S.E.B.M., 1966, v122.

Effect of Partial Hepatectomy and Pregnancy on Tumor Growth and Alanine-α-Ketoglutarate Transaminase Activity.* (31191)

HOMER R. HARDING,† FRED ROSEN, AND CHARLES A. NICHOL
Department of Experimental Therapeutics, Roswell Park Memorial Institute, New York State Department of Health, Buffalo, N. Y.

Characteristic of tumor growth is the competition between the neoplasm and normal tissues for the nutritional resources of the host(1,2). Emaciation and alterations in metabolism occur in animals bearing rapidly growing tumors. Previous studies in our laboratory demonstrated that hepatic alanine transaminase activity is markedly depressed in adult rats bearing Walker carcinoma 256 (3). It was of interest, therefore, to determine if this effect on a transaminase enzyme would also occur under other conditions which involve the growth of new tissues, such as during pregnancy and liver regeneration. The rate of tumor growth and changes in alanine transaminase activity were also observed when the Walker tumor was growing in competition with regenerating liver or rapidly growing fetal tissues. Furthermore, the induction of alanine transaminase by cortisol

* Supported in part by Grant CA-05671 from Nat. Cancer Inst.

† Present address: Sterling-Winthrop Research Institute, Rensselaer, N. Y.