4. Woodbury, D. M., Koch, A., Proc. Soc. Exp. Biol. & Med., 1957, v94, 720.
5. Lapland, J., Gargouil, Y. M., C. R. Soc. Biol., Paris, 1961, v155, 2439.
6. Draper, M. H., Friebel, H., Karzel, K., J. Physiol., London, 1963, v168, 1.
7. Boyle, P. J., Conway, E. J., J. Physiol., London, 1941, v100, 1.
8. Goldman, D. E., J. Gen. Physiol., 1943, v27, 37.
9. Hodgkin, A. L., Katz, B., J. Physiol., London, 1949, v108, 37.
10. Gourley, D. R. H., J. Pharmacol., 1964, v146, 307.
11. Galand, G., Ildefonse, M., J. Physiol., Paris, 1965, v57, 612.
12. Harris, E. J., Transport and Accumulation in Biological Systems, 2nd ed., Academic Press, New York, 1960.
13. Glynn, I. M., Pharmacol. Rev., 1964, v16, 381.

Received March 10, 1967. P.S.E.B.M., 1967, v125.

Stimulation of Interferon Production in Human Lymphocytes by Mitogens. (32235)

ROBERT M. FRIEDMAN AND HERBERT L. COOPER
(Introduced by A. S. Rabson)
National Cancer Institute and National Institute of Dental Research, National Institutes of Health, Bethesda, Md.

Recent studies have shown that a number of non-viral substances elicit the *in vitro* production of antiviral proteins with many of the properties of interferon (reviewed in ref. 1). One of the most interesting of these non-viral inducers of interferon (or interferon-like substances) is the phytohemagglutinin (PHA) obtained from the kidney bean, *Phaseolus vulgaris*. In human lymphocyte cultures PHA stimulates cellular enlargement and mitotic activity(2) and in addition initiates the production of an interferon-like protein(3). A variety of other substances are also capable of inducing lymphocyte growth *in vitro*. Some of these, like PHA, are non-specific, in that they are effective with cells from all subjects including cells obtained from umbilical cord blood(4). Other materials have been termed specific, since they are only active when the lymphocytes tested are obtained from a subject who has been immunologically sensitized to the substance in question(5,6).

It was of interest to determine whether the production of interferon by lymphocytes was a generalized concomitant of lymphocyte growth or whether the phenomenon was peculiar to stimulation by PHA. In this study the interferon-inducing effect of an extract of the poke weed (*Phytolacca americana*) which behaves as a non-specific mitogen(7) and that of streptolysin-O, which behaves as a specific mitogen(4), were investigated and compared with the effect of PHA. In addition, the kinetics of PHA-stimulated interferon production were studied in some detail.

Materials and methods. The preparation of purified human lymphocyte cultures has been previously described(8). Cells were cultured at a density of 2×10^6/ml in Eagle's MEM (spinner modification) with 10% autologous plasma.

Mitogens employed were PHA, poke weed mitogen (PWM), or streptolysin-O (SLO). PHA was obtained from Burroughs Wellcome, Inc. (PHA, batch x5) and was used at 10 $\mu g/10^6$ cells; SLO, from Difco Laboratories (0.4 ml standard solution/10^6 cells). Purified PWM was generously provided by Drs. L. Chessin, J. Börjeson, and R. Reisfeld, NIAID, NIH, and was employed at 1.25 $\mu g/10^6$ cells. PWM and PHA stimulated cultures were incubated for 2 to 48 hours before harvesting; SLO cultures, for 4-5 days. Since little or no mitotic activity was observed before 48 hours after addition of PHA or PWM, all of the observations made on those cultures were probably referable to the originally cultured cells.

All lymphocyte culture fluids to be assayed were routinely passed through Nalgene 0.2 μ

FIG. 1. Assay method for human interferon:

1. Mitogen added to human lymphocyte culture.
↓
2. Lymphocyte culture fluid harvested and filtered.
↓
3. Dilutions of lymphocyte culture fluid incubated for 14 hr with human adult skin cultures (HAS I cells).
↓
4. HAS I cells washed 5×, infected with virus for 1 hr; and again washed 5×.
↓
5. HAS I cells and culture fluids frozen and thawed during log phase of virus growth.
↓
6. HAS I culture fluids assayed for virus titer on chick fibroblasts.

plastic filter units and then diluted with Eagle's medium containing 10% fetal calf serum. To assay for antiviral activity (Fig. 1) test tubes containing monolayers of HAS I cells, a human adult skin diploid strain obtained from Dr. B. W. Uhlendorf, NIH, were treated for 14 hours with dilutions of human lymphocyte culture fluids. The monolayers were then washed 5 × with Eagle's medium and infected with a chick cell pool of Semliki Forest Virus (SFV) at a virus plaque forming unit to cell multiplicity of 80:1. Virus growth was interrupted by simultaneously freezing all of the tubes (—80°C) at one point in the log phase of virus growth, 5-9 hours after infection. Virus growth was assayed by titration of the frozen and thawed HAS I fluids on chick embryo monolayers and compared to virus growth in simultaneously run control tubes incubated with medium and serum before SFV infection. Fig. 2 shows the relationship between virus growth and dilutions of an interferon preparation obtained from SFV-infected HAS I cells. A linear relationship between the $\log_{10}$ of the interferon dilution and the percentage inhibition was found between 20 and 70% inhibition. A 3-fold or more reduction in virus growth was considered to be significant in this system. The titer of a preparation, therefore, was the highest dilution in which a 3-fold inhibition of virus yield was found. Most previous studies employing similar assay systems have considered a 2-fold reduction in virus growth as indicative of significant inhibition(9).

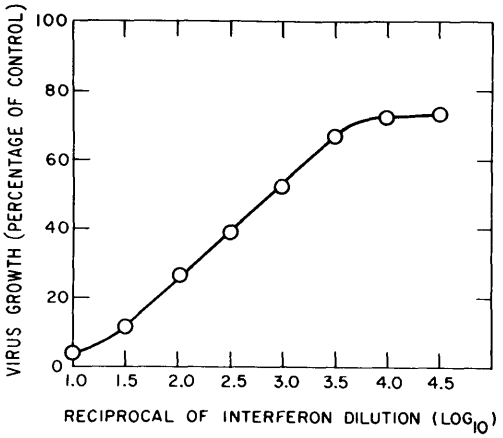

FIG. 2. Virus inhhibition by interferon produced in human cells. Interferon was produced by infecting human skin cultures with Semliki Forest Virus. Culture fluid was removed after 24 hrs, treated with acid to pH 2, neutralized, and diluted in Eagle's medium with 10% serum. Assay of dilutions for antiviral activity was performed by the method outlined in Fig. 1. Virus growth is reported as a percentage of that of control cultures.

Results. Table I reviews our results with cultures of unstimulated lymphocytes and with cultures stimulated by each of the 3 mitogens employed, SLO, PHA, or PWM. No antiviral activity was found in fluids from unstimulated lymphocyte cultures. Deinhardt and Burnside (personal communication) have reported low titers of interferon in some normal human leukocyte cultures but we have been unable to repeat these findings in our cultures which contained >98% lymphocytes. Since unstimulated lymphocyte cultures failed to produce antiviral activity, production of substances with anti-

TABLE I. Stimulation of Antiviral Activity in Human Lymphocytes by Mitogens.

Addition to lymphocytes*	Cultures producing antiviral activity /total tested	Range of positive cultures (units of antiviral activity/ml)
None	0/4	—
PHA, 1-2 days	10/11	100 to >100
SLO, 4-5 "	2/5	30
PWM, 1-2 "	5/6	30 to >100

* Lymphocyte cultures were incubated with 10 μg of phytohemagglutinin (PHA) per 10^6 cells; or, with 0.4 ml of a standard solution of streptolysin 0 (SLO) per 10^6 cells; or, with 1.25 μg of purified poke weed mitogen (PWM) per 10^6 cells for the indicated periods. Fluids were harvested, filtered, diluted with Eagle's medium with 10% fetal calf serum and assayed in human adult skin cultures as described in text.

viral activity in these systems after stimulation was probably not due to a latent virus infection.

In all but 1 of 11 experiments on lymphocytes from 11 different donors, high titers of antiviral activity were found in fluids from PHA stimulated lymphocyte cultures. Detectable titers were present as early as 2 hours after PHA addition. In SLO treated cultures 2 of 5 culture fluids had a moderate titer of antiviral activity after 4 days incubation. Also, no antiviral activity was noted in 2 additional cultures in which no mitotic actvity was present after SLO treatment. Finally, 5 of 6 PWM stimulated cultures had high titers of antiviral activity after 48 hours incubation with PWM. In culture fluids of a single additional PWM-treated culture which had no mitotic activity after incubation with PWM no antiviral activity was found.

The 3 SLO-treated cultures whch, despite typical mitotic responses to SLO, failed to produce an antiviral substance were of some interest. One of the cultures had been obtained from the same donor cell suspension from which the single negative in the PHA-stimulated group (Table I) had been; another, from the single negative in the PWM stimulated group (Table I). It is possible that certain cell suspensions were, for an unknown reason, poor producers of antiviral activity. Even some virus-cell systems which are usually excellent sources of interferon on occasion fail to produce significant interferon titers, *e.g.*, chikungunya virus-chick fibroblasts (S. Baron, personal communication).

Another possible reason for the relatively poor production of antiviral activity in SLO stimulated cells may be related to the fact that the mitotic response to SLO was of lower degree and of more gradual onset than that to either PHA or PWM(5). This may be due to the requirement for a series of divisions of a small, SLO-sensitive segment of the lymphocyte population, in response to specific antigens before the mitogenic response could be detected. Since, as will be shown below, interferon production was greatest shortly after exposure to the stimulating agent and decreased thereafter, it may be that the total production in some SLO-stimulated cultures fell below the limits of detection by our assay system because of an initially small sensitive population.

TABLE II. Biological and Physical Properties of Antiviral Substance Produced in Human Lymphocyte Cultures Exposed to Phytohemagglutinin, Streptolysin 0, or Poke Weed Mitogen.

Treatment	Antiviral activity
Acid to pH=2	Preserved
44°C, .5 hr	"
60°C, .5 hr	Destroyed
Trypsin (200 μg/ml, 37°, 15 min)	"
105,000 × *g*, 2 hr	Not sedimented
Washing cells 5× after incubation	Not removed
On human cells	Present
On chick cells	Absent

The antiviral substance produced by human lymphocyte cultures treated with PWM, PHA, or SLO had many properties in common with those of human interferon(10). It was stable to acid (pH=2) and to 44° for 0.5 hour, but not to 60°C for 0.5 hour or to trypsin treatment. It was not sedimented by centrifugation at 105,000 × *g* for 2 hours. The antiviral activity was not removed by washing the HAS I cells 5 × after incubation with lymphocyte culture fluids and was species specific, since it protected human cells but not chick cells against SFV (Table II). In addition Merigan (personal communication) has shown that the antiviral substance produced by human lymphocytes in response to PHA has the same molecular weight as purified human interferon (about 18,000). In all chemical, physical, and biological properties so far investigated in our laboratory the properties of the antiviral substances produced in human lymphocytes in response to all 3 mitogens were identical to those of human interferon. Wheelock(3) has reported that the antiviral substance induced in human lymphocytes by PHA was not stable to pH 2; however, Merigan and Strander and Cantell (personal communications) have found that it was stable, corresponding to our findings. Otherwise, the properties reported here agree with those reported by Wheelock for PHA induced interferon(3).

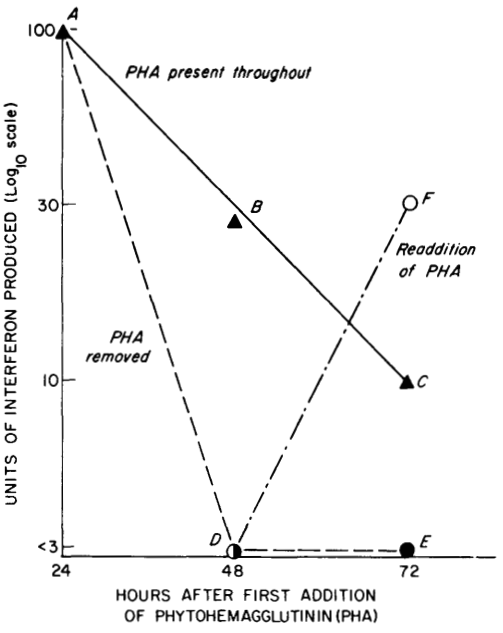

FIG. 3. Effect of removal and readdition of phytohemagglutinin (PHA) on interferon production in human lymphocyte cultures. A, B, and C—PHA (10 μgm/10^6 cells) present 24, 48, and 72 hrs respectively. D, E—PHA removed after 24 hrs. F—PHA again added after 24 hrs incubation in its absence. All results represent interferon produced in a 24 hr period.

Since PHA may act as an inducer in the stimulation of interferon production, it was of interest to determine whether continuation of interferon production was dependent on the continued presence of PHA in the culture medium. It has already been shown that the mitogenic response of lymphocytes does not require the continuous presence of PHA after an initial exposure(11). The experiment summarized in Fig. 3 was therefore performed. Cultures were exposed to PHA. One group was washed twice with medium every 24 hours and PHA was added in the replacement fluids. Antiviral activity was present in these culture fluids up to 72 hours after first addition of PHA (Points A, B, and C, Fig. 3), but titers diminished in successive 24-hour harvests. In another group, PHA was removed by washing after 24 hours and not replaced. Interferon production, which had been at high levels during the initial 24 hours of exposure to PHA fell below detectable levels during the succeeding 48 hours without PHA (D&E, Fig. 3). Indeed, it was found that interferon production had ceased by 2 hours after PHA had been removed. When PHA was replaced 48 hours after the first PHA addition and after 24 hours incubation in its absence, moderate titers of interferon were produced in the next 24 hours (F, Fig. 3).

Discussion. The results confirmed those of Wheelock(3) and in addition indicated that several mitogens were capable of inducing interferon production in human lymphocytes. In the case of all mitogens tested this response was variable but in all instances where very little or no mitotic activity had been induced, no interferon production was found. It is apparent that interferon production was not a peculiarity of lymphocyte interaction with PHA, but was more generally related to lymphocyte growth stimulation. Since the greatest production of interferon occurred during the initial 24 hours of PHA-stimulated growth and thereafter declined, and since significant DNA synthesis and mitosis did not begin in this system until 40-48 hours had elapsed, interferon production appeared to be related to events occurring in the G1 phase of the mitotic cycle.

The findings also showed that upon removal of PHA from lymphocytes interferon synthesis ceased within 2 hours and with readdition of PHA interferon production was again initiated. This suggested the existence in human cells of a specific, relatively short-lived messenger RNA. Alternatively, translational repression may have become effective within 2 hours of PHA removal. Resumption of interferon production with readdition of PHA may then have resulted from derepression at either the transcriptional or translational level. The data presented, however, do not rule out the possibility that the interferon production which occurred upon readdition of PHA was due to stimulation of a previously unaffected segment of the cell population.

Summary. Phytohemagglutinin (PHA), poke weed mitogen, and streptolysin-O, all agents capable of stimulating mitosis in human lymphocytes *in vitro* also stimulated the production of an antiviral substance

with biological, chemical, and physical properties identical to those of human interferon. Upon removal of PHA from human lymphocyte cultures interferon production ceased within 2 hours. Readdition of PHA resulted in reinitiation of interferon production.

1. Ho, M., Fantes, K. H., Burke, D. C., Finter, N. B., in Interferons, N. B. Finter, ed., North Holland, Amsterdam, 1966, p181.
2. Nowell, P., Cancer Res., 1960, v20, 462.
3. Wheelock, E. F., Science, 1965, v149, 310.
4. Hirschhorn, K., Schreibman, R., Verbo, S., Gruskin, R., Proc. Nat. Acad. Sci. USA, 1964, v52, 1151.
5. Pearmain, G., Lycette, R., Fitzgerald, P., Lancet, 1963, vi, 637.
6. Elves, M., Roath, S., Israels, M., ibid., 1963, vi, 805.
7. Farnes, P., Barker, B., Brownhill, L., Fanger, H., ibid., 1964, vii, 1100.
8. Cooper, H., Rubin, A., Science, 1966, v152, 516.
9. Isaacs, A., Adv. in Virus Res., 1963, v10, 1.
10. Merigan, T. C., Gregory, D. F., Petralli, J. K., Virology, 1966, v29, 515.
11. Newsome, J., Lancet, 1963, vii, 91.

Received March 13, 1967. P.S.E.B.M., 1967, v125.

Neomycin Inhibition of Lipolysis *in vitro*.* (32236)

SATISH K. MEHTA,† ELLIOT WESER,‡ AND MARVIN H. SLEISENGER
(Introduced by A. G. Bearn)
Division of Gastroenterology, Department of Medicine, Cornell University Medical College, New York City

Oral administration of neomycin may increase fecal fat excretion in normal human subjects(1). Histologic changes of the jejunal mucosa resulting from this antibiotic were reported by Jacobson, Prior and Faloon(2), but were not confirmed by Rubin and Dobbins(3). In a more recent study, defective hydrolysis of a long-chain unsaturated fat was demonstrated in 2 adults receiving oral neomycin(4). For this reason, the effect of neomycin and other antibiotics on pancreatic lipolysis was studied *in vitro*.

Methods. Pancreatic juice was collected at room temperature from a pancreaticocutaneous fistula in an asymptomatic non-fasting 32 year old white male. Injection of radiopaque material revealed that the fistula communicated directly with the main pancreatic duct. The colorless, slightly turbid, alkaline fluid (amylase activity 947 Somogyi units per ml) was centrifuged for 15 minutes at 2,500 g and 4°C, lyophilized, and stored in a vacuum desiccator at 4°C. Just prior to use, the lyophilized powder was dissolved in 1 ml of cold 0.05 M Tris (Trihydroxymethyl aminomethane) buffer, pH 7.0, and the final concentration of protein adjusted to 2 mg per ml. Protein was measured by the method of Warburg and Christian(5).

Lipolysis was measured at room temperature by a modification of the turbidimetric method of Vogel and Zieve(6). The triglyceride substrate was a stable emulsion of olive oil (0.04%) and sodium taurocholate (0.4%) in 0.05 M Tris buffer, pH 7.0. The reaction was started by adding 20 to 43 μg of pancreatic fluid protein to 2.8 ml of the olive oil emulsion. After inverting the mixture 3 times, lipolytic activity, reflected by clearing of the mixture, was measured by the change in absorbancy per minute between 2 and 5 minutes. Absorbancy was recorded in a 3 ml quartz cuvette with a 1 cm light path using a Zeiss spectrophotometer set at 650 mμ. No change in absorbancy was noted in a control cuvette when an equal volume of buffer without pancreatic enzyme was added to olive oil emulsion. The pH of the assay mixture remained constant during the initial

* This investigation was supported by N.I.H. grant CA-09386 1051 GM, and the John A. Hartford Foundation.

† Present address: Post Graduate Medical Research Institute, Chandigarh Punjab, India.
‡ Present address: Univ. of Texas, South Texas Med. School, San Antonio.