Immune Specific Induction of Interferon Production in Cultures of Human Blood Lymphocytes

Abstract. *Human blood lymphocytes stimulated with nonviral antigens in vitro produce an antiviral substance with the biological and biochemical characteristics of interferon. The induced response was specific for cells obtained from immune donors. Cells from nonimmune donors did not produce interferon on exposure to these substances. The quantity of interferon produced by antigen stimulation was related to concentration of antigen over a relatively narrow range; with higher concentrations induction was decreased. Interferon production was maximum during days 4 to 7 in culture. In contrast, phytohemagglutinin-induced interferon was primarily produced during the first 4 days in culture.*

The role of interferon as part of the host defenses during primary viral infection has been well established (*1*). However, scant attention has been paid to this host response during reinfection. The close relation between the immune response and resistance to reinfection has suggested that protection against reinfection with a viral agent is predominantly a function of antibody production (*2*) or cellular hypersensitivity (*3*) or both. Glascow has shown that cultures of peritoneal leukocytes from mice sensitized to Chikungunya virus will respond to this agent by increased interferon production (*4*). His data indicate that immune recognition mechanisms are involved in this enhanced interferon response. We have examined the relation between interferon production and the immune response to various nonviral antigens. Our studies show that nonviral antigens may induce interferon production in lymphocyte cultures from sensitized human donors and that these antigens do not induce interferon production in cells from nonimmune human donors.

Human peripheral blood lymphocytes were prepared as described (*5*). Cultures were established with 1.0×10^6 lymphocytes per milliliter and initially contained a variable number of neutrophiles (20 to 40 percent) and monocytes (1 to 2 percent). These cells were maintained in medium 199 with Earle's salts and 20 percent inactivated calf serum, 100 units of penicillin per milliliter, and 100 μg of streptomycin per milliliter. Within 48 hours, 95 to 99 percent of the cells consisted of small lymphocytes.

Antigens used in this study consisted of purified protein derivative of tuberculin (PPD) (*6*), and highly purified tetanus toxoid and diphtheria toxoid (*7*). Doses of PPD were generally from 0.2 to 10.0 μg/ml; and doses of diphtheria and tetanus toxoids were 5 to 100 Lf (limit of floculation) unit/ml. These ranges were used because they had been found to be effective inducers of DNA synthesis (*8*). Cultures containing phytohemagglutinin M, Difco (PHA), were included in all experiments as positive controls, so that the ability of cells to produce interferon in response to a known "nonviral" inducer could be assessed (*9*). Such cultures also provided a base for comparison of the magnitude of the cellular response to the various antigens. The PHA was used at a concentration that stimulated maximum interferon production. Antigens were added at the time the cultures were prepared, and the cells were then maintained for varying periods up to 10 days at 36°C in a humidified atmosphere containing 5 percent CO_2. Control leukocyte cultures, without stimuli, were similarly prepared and maintained. All cultures were established in duplicate. At the end of the incubation period (4 to 10 days), the cultures were centrifuged at 300*g* for 5 minutes, and the supernatants were separated and saved. Immune sensitization of the donors was ascertained by skin test for tuberculin (Mantoux, first strength) and by history and medical records for diphtheria and tetanus toxoids.

Stationary monolayer cultures of primary human amnion cells (*10*) were used to assay for interferon. Maintenance medium consisted of 45 percent Hanks solution, 45 percent bovine amniotic fluid, 5 percent beef embryo extract, and 5 percent inactivated horse serum. All media contained (per milliliter) 100 units of penicillin, 100 μg of streptomycin, and 5 μg of amphotericin B. Only confluent cell sheets were used for assay. Leukocyte culture supernatants undiluted and in twofold serial dilutions were added in 0.5-ml amounts to duplicate tube cultures of human amnion cells. The tubes were then placed on a roller wheel to assure even exposure of the cells to the inoculum. After 18 to 24 hours, the inocula were decanted, the cell monolayers were rinsed with 1 ml of sterile neutralized Hanks solution, and 100 to 1000 $TCID_{50}$ (tissue culture infective doses, 50 percent effective) of either Sindbis or vesicular stomatitis virus were added in 1 ml of maintenance medium. Cultures were then incubated in stationary racks and examined for cytopathologic effect (CPE) at daily intervals. Interferon was present in those cultures in which fewer than 25 percent of the cells showed CPE at the time when nearly all cells in control cultures showed CPE. A single unit of interferon was defined as the reciprocal of the end-point dilution.

Diphtheria toxoid, tetanus toxoid, and PPD were added to lymphocyte cultures from immune and nonimmune donors. The cultures were incubated for

Table 1. Relation between immunologic history and interferon production in cultures of human blood lymphocytes. TT, tetanus toxoid; DT, diphtheria toxoid.

Immunologic history		Units of interferon produced in response to:				
Interval between* booster and culture	Tuberculin skin test	No stimulus	PHA	TT	DT	PPD
5 days		0	128	16	8	
7 days		0	32	32	8	
	+	0	32			4
	+	0	8			4
2 years		0	16	4	0	
	+	0	8			64
	+	0	64			128
	+	0	2			8
	+	2	32			64
4 years	+	2	64	8	8	32
10 years	−	0	4	0	0	0
2 years	−	0	4	0	0	0
	−	0	16			0
1 year	−	0	16	4		0
6 months	−	0	32	8		0
20 months	−	2	16	0	0	0

* The booster was diphtheria and tetanus toxoids.

4 days in the presence of these antigens, and the supernatants of the cultures were then examined for interferon. Antiviral activity of the culture supernatants was directly related to the donor's immunologic sensitivity to the specific test antigens. Thus, cells from individuals recently immunized with tetanus and diphtheria toxoids (*11*) produced interferon when cultured with these specific antigens; cells from donors not recently immunized with these toxoids failed to produce significant titers of interferon (see Table 1). Addition of PPD also evoked the production of antiviral activity in lymphocyte cultures from sensitive donors but not in those from unsensitized donors. Phytohemagglutinin regularly produced interferon in cells from all donors, and the quantities produced were similar to those obtained by immune stimuli. Unstimulated cultures generally did not produce interferon.

The relation between the concentration of antigen present in the medium and the interferon response of antigen-sensitive cells was next examined. For these and subsequent experiments, PPD was employed as antigen since the immunologic status of our donors with regard to tuberculin could be more clearly defined than with diphtheria or tetanus toxoids. Lymphocyte cultures were exposed for 4 days to varying concentrations of PPD, and the supernatants were then examined for interferon activity. This interval was selected after lymphocyte cultures, incubated with PHA, were found to produce peak interferon titers within 4 days. In a typical experiment (Fig. 1), two donors were used—one "skin-test" positive to PPD ("immune") and one "skin-test" negative ("nonimmune"). Although the cells from both donors produced interferon in response to PHA, only cells from the sensitized donor responded to PPD stimulation. The quantity of interferon induced by PPD increased with PPD concentration, reaching a maximum at 3 μg/ml. In this particular experiment, the titers of interferon induced by the optimum PPD concentration exceeded the titers resulting from stimulation with PHA. With PPD concentrations in excess of the optimum, interferon production was decreased. The optimum concentration of antigen necessary to evoke maximum interferon synthesis was found to vary somewhat with individual donors and ranged from 2 to 3.5 μg/ml.

Replicate cultures of lymphocytes

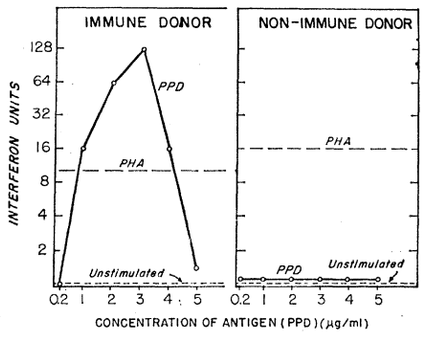

Fig. 1. The effect of antigen (PPD) concentration on the production of interferon by cultures of lymphocytes from immune and nonimmune donors. Interferon assays were done on culture supernatants collected on day 4.

from immune and nonimmune donors were established in media containing 3.0 μg of PPD per milliliter. Similar cultures with and without PHA were also included as controls. At intervals during the following 10-day period, duplicate cultures representing each of these variables were terminated, and their supernatants were removed and stored at −20°C for simultaneous interferon assay (Fig. 2). Cells from immune and nonimmune donors, incubated with PHA, produced similar amounts of interferon. In addition, small quantities of interferon were found in unstimulated cultures of both immune and nonimmune cells. Interferon titers in these latter cultures were not generally demonstrable before day 4. Highest titers were obtained in lymphocyte cultures from the sensitized donor when these cells were exposed to antigen (PPD). In the antigen-stimulated cultures, the maximum period of inter-

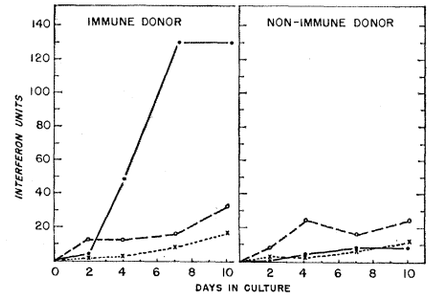

Fig. 2. Interferon production by lymphocyte cultures from immune and nonimmune donors. The response of unstimulated cultures is compared with that due to PHA and antigen (PPD). Interferon assays were done on culture supernatants collected on days 2, 4, 7, and 10. ●——● PPD, 3 μg/ml; ○- - - -○ PHA, 0.3 ml/ml; x- - - -x, unstimulated.

feron production was from days 4 through 7. The same antigen added to cultures from nonimmune donors did not stimulate interferon production beyond that observed in unstimulated controls.

The antiviral factor produced by stimulation of the lymphocyte cultures fulfilled the criteria commonly accepted for interferon. It was stable on dialysis against buffers at *p*H 4 to 10 at 4°C for 24 hours; it did not sediment in the ultracentrifuge at 100,000*g* for 2 hours; it was resistant to deoxyribonuclease and ribonuclease; it had activity in cells of human origin against a number of different viruses, including poliovirus type II, vesicular stomatitis virus, Sindbis virus, and vaccinia virus. Its effect was species-specific for cells of human origin only; it did not protect L cells (mouse), chick embryo fibroblasts, or rabbit kidney cells against these agents. Activity was lost upon incubation of the undiluted leukocyte culture supernatant with 1.25 mg of trypsin per milliliter at *p*H 7.6 to 7.8 for 5 hours at 37°C. In addition, this factor had no direct effect on the viruses tested.

A number of authors have shown that human leukocytes in culture can produce interferon in response to viral agents (*12*). Interferon production in leukocyte cultures has also been demonstrated with several nonviral agents, including PHA and other mitogens (*9*, *13*). Glascow has found that cultures of peritoneal leukocytes from mice immunized with Chikungunya virus produce 2 to 10 times more interferon than control cells obtained from mice without previous immunologic experience, when both preparations were exposed to this virus (*4*). Our studies have expanded these observations by demonstrating that interferon production can be induced by nonviral antigens in cultures of human peripheral lymphocytes. This effect is dependent upon the prior immunological history of the donor. Thus, immune recognition mechanisms may generally function as inducers of interferon.

A marked quantitative difference exists between the degree of blastogenesis and the amount of interferon induced by PHA and antigen in lymphocyte cultures. The addition of PHA to such cultures results in the production of 70 to 90 percent of blastoid cells, as compared with less than 10 percent for antigen stimulation (*8*). In our experiments, PHA-stimulated cultures often yielded smaller quantities of inter-

feron than those produced in similar cultures stimulated with antigen. Despite the apparent lack of relation between the degree of blastogenesis and interferon production, our data suggest that only those concentrations of PHA or antigen which stimulate blastoid formation are effective inducers of interferon. Maximum synthesis of antigen-induced interferon occurred between 4 to 7 days in culture, while maximum production of PHA-induced interferon occurred within the first 4 days in culture. These intervals correlate with the appearance of blastoid cells in these cultures.

A previous study with viruses has indicated that immune recognition mechanisms may generally function as an enhancer of interferon production (*4*). Our study with nonviral antigens shows that such mechanisms may also function as inducers of interferon. It has not yet been shown that this mechanism is also operative in vivo. Our observations, however, indicate that interferon induction is part of the immune response. These results suggest that the immune induction of interferon may also have a role in vivo.

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Cell Division: A Second Circadian Clock System in Paramecium multimicronucleatum

Abstract. *Light-dark cycles entrain a pattern of division in a population of individually isolated cells cultured in excess nutrients at 14°C. This pattern persists for at least 8 days in continuous dim light. Both clones which do and which do not express a circadian rhythm of mating-type reversals can be entrained. The phase is a clonal characteristic.*

Acyclic cells in *Paramecium multimicronucleatum*, syngen 2, are homozygous for a recessive allele (*c*) and possibly represent a mutant without a circadian clock. They do not express the circadian rhythm of mating-type reversals that cyclic cells with the dominant allele (*C*) express (*1*). Because circadian rhythms of cell division have been reported in a number of Protista (*2*), an assay for a rhythm of cell division in this system was developed. Cell division is used as a second circadian rhythm to help delimit the point of the genetic block.

Cells were assayed by making periodic observations on a fixed population of 118 cells individually isolated in excess food and at a constant temperature (14°C). Temperature, which limits the number of divisions to less than one a day but has no influence over time of division, was controlled by constantly circulating water through a miniature plexiglass waterbath from a reservoir monitored by a thermoregu-

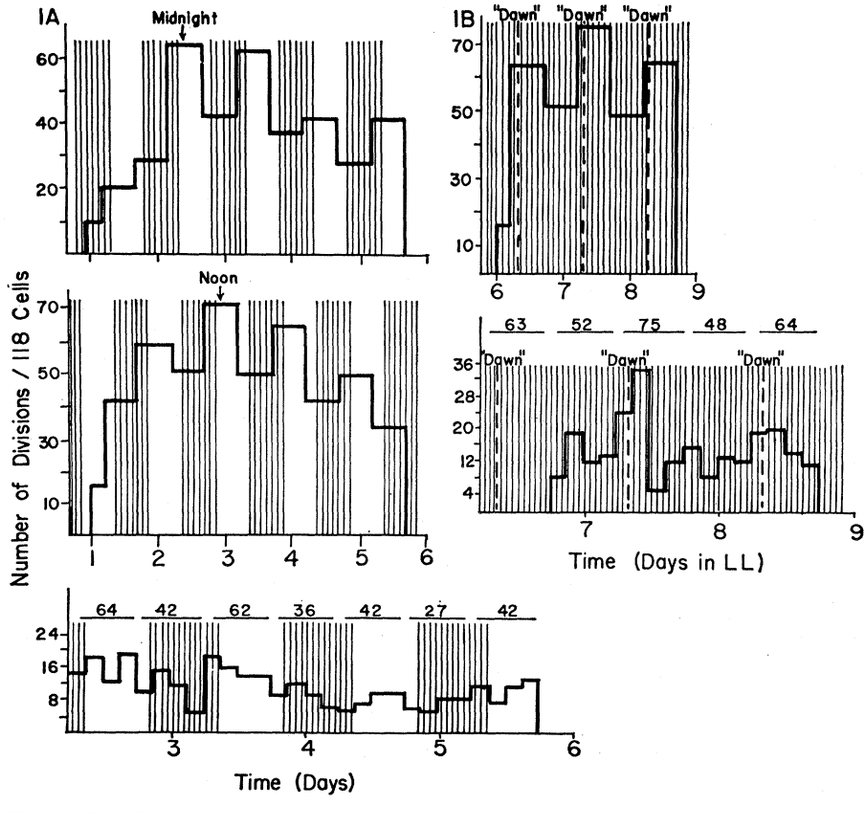

Fig. 1. Distribution of cell divisions in cyclic clone d2-1 of *P. multimicronucleatum* growing in excess nutrients at 14°C. Cells which had been growing in mass culture on the light–dark (LD) regime were individually distributed into 118 wells starting at 1400 (EDT). Counts were made, and one daughter cell of each fission was removed every 3 hours. White represents daylight (< 600 lux), and shading represents the night-light (< 60 lux). (A) Entrainment response in two regimes of LD 12 : 12 (600 lux : 60 lux) which differ by 12 hours in the time of the daily onset (1000, center; or 2200, upper chart) of daylight. The 3-hour counts, totaled from 0800 to 2000 and from 2000 to 0800, are summarized in the top two charts. The bottom chart shows the actual 3-hour counts and the 12-hour total (above the bar) for the top chart. (B) Circadian rhythmicity of cell division for the last 3 of 8 days' growth in constant dim (60 lux) light (LL) after the entrainment seen in A (center). Counts plotted as in (A).