PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 154, 138-141 (1977)

Relative Ability of Mitogens to Stimulate Production of Interferon by Lymphoid Cells and to Induce Suppression of the *in Vitro* Immune Response (39622)

HOWARD M. JOHNSON, G. JOHN STANTON, AND SAMUEL BARON

Department of Microbiology, The University of Texas Medical Branch, Galveston, Texas 77550

T lymphocyte mitogens are excellent inducers of suppressor cell activity in mouse lymphoid cell cultures (1-4). An array of lymphokines, including interferon, is produced by lymphocyte cultures that have been stimulated by these mitogens (5-7). In the mouse system, mitogen-induced (mitogen-type) interferon is antigenically different from virus-induced (virus-type) interferon (7), and may be identical or related to antigen-induced (antigen-type) interferon (8). It is becoming clear that, depending upon the inducer and the stimulated cell, there are several types of interferons that can be produced by mammalian cells. The following is defined for operational purposes:

1. *Virus-induced (virus-type) interferon.* This term is used for interferon produced in lymphoid or nonlymphoid cells following stimulation by virus or double-stranded RNA. This interferon is sometimes called type I interferon in the murine system.

2. *Mitogen-induced (mitogen-type) interferon.* This term is used for interferon produced in lymphoid cells following stimulation by T lymphocyte mitogens. This interferon is antigenically distinct from virus-induced (virus-type) interferon.

3. *Antigen-induced (antigen-type) interferon.* This term is used for interferon produced in antigen-primed lymphoid cells upon second exposure to the specific antigen. This interferon is also antigenically distinct from virus-induced (virus-type) interferon, and is sometimes called type II interferon in the murine system. It is tentatively felt that mitogen- and antigen-type interferons are the same substances in the murine system since no evidence to the contrary is presently available.

We are interested in the possible role of mitogen-induced interferon in the mediation of mitogen-induced suppressor cell activity. Several T cell mitogens are compared here, then, for their relative ability to suppress the *in vitro* plaque-forming cell response of mouse spleen cells, and to stimulate the production of interferon in mouse spleen cell cultures. Concanavalin A, phytohemagglutinin P, and staphylococcal enterotoxin A are the T cell mitogens examined. This report will show that the relative abilities of these substances to stimulate interferon in the cultures are quantitatively related to their ability to induce the suppressor cell state.

Materials and methods. Mice. C57B1/6 female mice were obtained from the Laboratory Supply Company, Indianapolis, Ind.

Sheep red blood cells (SRBC). SRBC (9) were obtained from the Colorado Serum Company, Denver, Colo. The SRBC used throughout the study were obtained from a single sheep (No. 446).

Diluent. Spleen cells at the time of harvesting, SRBC, mitogens, and interferon were all suspended or diluted in Eagle's minimal essential medium (MEM) containing spinner salts, but no L-glutamine or sodium bicarbonate (9).

Mitogens. Concanavalin A (Con A), twice crystallized, was obtained from ICN Pharmaceuticals, Cleveland, Ohio. It was stored at room temperature. Substocks diluted in modified MEM were stored at $-70°$. Staphylococcal enterotoxin A (SEA) was produced by the Microbial Biochemistry Branch, Division of Microbiology, Food and Drug Administration, Cincinnati, Ohio (10). Its purity was estimated to be >99% by extinction coefficient (11). Purified phytohemagglutinin P (PHA-P) was obtained from Difco Laboratories, Detroit, Mich. Substocks were diluted in modified MEM and stored at $-20°$.

Cultures. Dissociated normal mouse spleen cells were cultured for *in vitro* anti-SRBC plaque-forming cell (PFC) response exactly as described by Mishell and Dutton

(9). Cultures consisted of 1 ml of 1.5×10^7 spleen cells and 3×10^6 SRBC. All PFC responses were determined at Day 5. Cell viabilities were determined by trypan blue dye exclusion. Direct PFC assays were performed on microscope slides as previously described (12). A single lot (C640521) of fetal calf serum, obtained from Gibco Diagnostics, Grand Island, New York, was used throughout the study. All results are expressed as the average of duplicate cultures.

Mitogen induction of interferon. Mouse spleen cell cultures were prepared exactly as described above for *in vitro* PFC response except for the absence of SRBC. Various concentrations of the mitogens were added to the cultures and the cells were routinely incubated for 48 hr under the above described conditions (9). Supernatant fluids were obtained by centrifugation of the harvested cultures at 1000 rpm in an RC-3 Sorvall centrifuge at 7°. These fluids were assayed on the day of harvest for mitogen-induced interferon activity in mouse L cells by a slight modification of the method of inhibition of cytotopathic effect of vesicular stomatitis virus (100 $TCID_{50}$/challenge dose) (13).

Results and discussion. The relative abilities of various concentrations of Con A, PHA-P, and SEA to inhibit the PFC response when added to cultures at the same time as antigen are shown in Fig. 1. Coefficients of variation for duplicate determinations were generally less than 20%. SEA was the most effective inhibitor, 0.01 μg/ml resulting in 88% inhibition of the PFC response, while 0.1 μg/ml and greater concentrations caused >99% inhibition of the anti-SRBC PFC response. Con A was the second-most effective inhibitor, with 1.25 μg/ml inhibiting the PFC response by 78%. Concentrations of 2.5 μg/ml or greater resulted in >90% inhibition of the anti-SRBC PFC response. Interestingly, 2.5 μg/ml of Con A was more inhibitory than 5 and 10 μg. This pattern of inhibition was observed in repeated experiments. Con A was nontoxic to spleen cells up to 2.5 μg/ml. Slight toxicity was observed at 5 and 10 μg of Con A. PHA-P was the least effective inhibitor of the PFC response; 10 μg/ml was required for 76% inhibition.

Representative data demonstrating the relative abilities of Con A, PHA-P, and SEA to stimulate mouse spleen cell cultures to produce mitogen-type interferon are presented in Fig. 2. Coefficients of variation for duplicate determinations were generally less than 25%. The interferon assays were carried out in cultures separate from those used for PFC responses, because of the differences in incubation times of the two systems. The data in Figs. 1 and 2 are evalu-

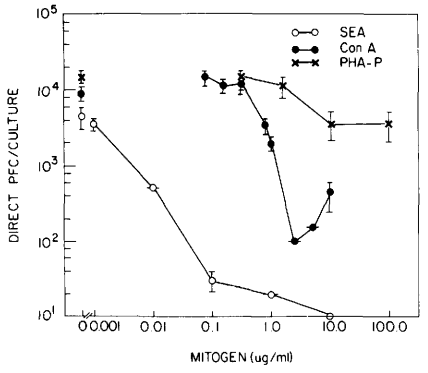

FIG. 1. The suppressive effect of various T lymphocyte mitogens on the primary *in vitro* PFC response to SRBC. Mitogens were added at the time of SRBC addition, and direct anti-SRBC PFC/culture was determined on Day 5. PFC responses are expressed as the mean of duplicate determinations ± SD. The responses are representative of three experiments.

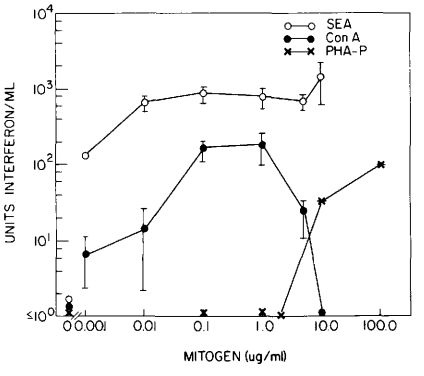

FIG. 2. Stimulation of the production of mitogen-type interferon in C57B1/6 mouse spleen cell cultures by various T lymphocyte mitogens. Spleen cells and mitogens were incubated for 48 hr under conditions as described for the PFC response. Interferon concentrations are expressed as the mean of duplicate determinations ± SD. The SD (not plotted) for 0.001 μg of SEA is 146. The responses are representative of three experiments.

ated, then, for broad correlations at various mitogen concentrations, rather than at a specific mitogen concentration. SEA was the most effective stimulator of interferon; 134 NIH reference units of interferon/ml were produced at 0.001 μg/ml and 644 to 1323 units/ml at 0.01 to 10 μg of SEA/ml. Con A stimulated the cultures to produce 150 and 189 units of interferon/ml at 0.1 and 1.0 μg/ml, respectively. At 0.01 μg of Con A/ml, only 13 units of interferon were produced. At 5 and 10 μg of Con A/ml, the amount of interferon produced significantly declined. This was probably due to the fact that Con A was cytotoxic at these concentrations. The Con A interferon data are consistent with the slight recovery of the PFC response (Fig. 1) at the higher concentrations of Con A. PHA-P was the least effective inducer of interferon; 10 μg/ml stimulated the cultures to produce 32 units of interferon/ml, and 100 μg stimulated 100 units. Lesser concentrations of PHA-P did not stimulate the production of interferon in the cultures. SEA was the most effective inhibitor of the PFC response and was the best inducer of mitogen-type interferon, followed by Con A, with PHA-P being the least effective. This was generally observed both in terms of the concentration of mitogen required for the observed biological activity, and for the extent of the measured activity. Repeated experiments resulted in the same response patterns as illustrated in Figs. 1 and 2, although absolute values varied between experiments.

Kinetic studies (Fig. 3) demonstrated that SEA induction of interferon was substantial at 48 hr of culture, the incubation time used for the data of Fig. 2. A further increase in interferon activity was sometimes observed on Days 3 and 4 of culture. Occasionally a decline in interferon activity was observed on the fifth day of mitogen stimulation of spleen cultures (data not shown). The kinetics of interferon production by SEA-stimulated cultures are similar to those observed for Con A- and PHA-P-stimulated cultures (14). A 48-hr incubation time appears suitable, then, for assessing the relative abilities of these mitogens to stimulate interferon production.

The mitogen-induced interferons are species specific (7, 8). They did not exhibit antiviral activity in human WISH cell lines. It has previously been demonstrated that the mitogen-type interferon is antigenically distinct from virus-type interferon (7, 8); antibodies to virus-type interferon blocked the antiviral activity and PFC inhibitory activity of virus-type interferon, while having no inhibitory effect on mitogen-type interferon.

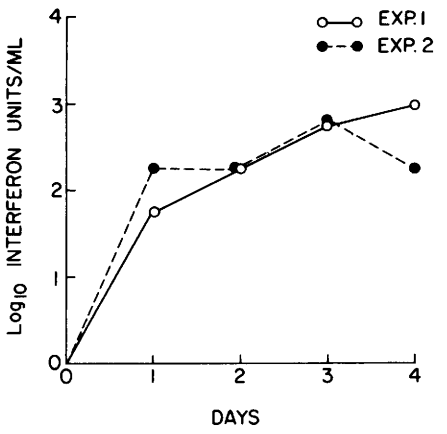

FIG. 3. Kinetics of SEA stimulation of interferon production in C57B1/6 mouse spleen cell cultures. SEA was used at 0.5 μg/ml. The SD (not shown) is similar to those of interferon assays in Fig. 2.

The data are consistent with the interpretation that mitogen-type interferon might be the mediator of mitogen-induced suppressor cell activity. Further, it is possible that mitogen-type interferon, mitogen-induced macrophage migration inhibitory factor (MIF), and mitogen-induced soluble immune response suppressor (SIRS) may be biological expressions of the same substance (7, 8, 15, 16). It has not been possible to separate MIF and antigen-induced interferon (8). Neither has SIRS activity been separated from MIF activity (15). The interferon assay system, as demonstrated here, may be a convenient technique for quantitating these biological activities.

Prerequisite to purification of mitogen-type interferon for characterization and immunization for specific antibodies is a determination of the most suitable inducers. We have shown here that SEA is the most potent inducer of mitogen-type interferon and that this inducer is highly active over a wide

range of concentrations. The data presented here are consistent with and supportive of previous studies indicating a relationship between mitogen-type interferon and regulation of the immune response by suppressor T cells (7).

Summary. Several T cell mitogens, concanavalin A (Con A), phytohemagglutinin P (PHA-P), and staphylococcal enterotoxin A (SEA), were compared for their ability to inhibit the *in vitro* antibody response and to stimulate the production of mitogen (antigen) -type interferon in mouse spleen cell cultures. It was found that the ability to inhibit the plaque-forming cell (PFC) response to sheep red blood cells was proportional to the ability of these mitogens to induce interferon in the cultures. SEA was the most effective inhibitor of the PFC response and the best inducer of mitogen-type interferon, followed by Con A, with PHA-P being the least effective. The data suggest that SEA would be the most suitable inducer of mitogen-type interferon in quantity as a prerequisite to purification and characterization of the molecule. The data are supportive of previous studies suggesting a role for mitogen (antigen) -type interferon in regulation of the immune response via suppressor T cells.

We wish to thank Ms. Phyllis Myers and Mr. Kley Hughes for their skillful technical assistance.

1. Dutton, R. W., J. Exp. Med. **136,** 1445 (1972).
2. Rich, R. R., and Pierce, C. W., J. Exp. Med. **137,** 205 (1973).
3. Watson, J., Epstein, R., Nakoinz, I., and Ralph, P., J. Immunol. **110,** 43 (1973).
4. Smith, B. G., and Johnson, H. M., J. Immunol. **115,** 575 (1975).
5. David, J. R., N. Engl. J. Med. **288,** 143 (1973).
6. Merigan, T. C., *in* "Interferons and Interferon Inducers" (N. B. Finter, ed.), pp. 65–68. North-Holland/American Elsevier, New York (1973).
7. Johnson, H. M., and Baron, S., Cell. Immunol. **25,** 106 (1976).
8. Youngner, J. S., and Salvin, S. B., J. Immunol. **111,** 1914 (1973).
9. Mishell, R. I., and Dutton, R. W., J. Exp. Med. **126,** 423 (1967).
10. Bergdoll, M. S., *in* "Microbial Toxins" (T. C. Montie, S. Kadis, and S. J. Ajil, eds.), Vol. III, p. 265. Academic Press, New York (1970).
11. Schantz, E. J., Roessler, W. G., Woodburn, M. J., Lynch, J. M., Jacoby, H. M., Silverman, S., Gorman, J. C., and Spero, L., Biochemistry **11,** 360 (1972).
12. Golub, E. S., Mishell, R. I., Weigle, W. O., and Dutton, R. W., J. Immunol. **100,** 133 (1968).
13. Armstrong, J. A., Appl. Microbiol. **21,** 723 (1971).
14. Stobo, J., Green, I., Jackson, L., and Baron, S., J. Immunol. **112,** 1589 (1974).
15. Rich, R. R., and Pierce, C. W., J. Immunol. **112,** 1360 (1974).
16. Tadakuma, T., Kuhner, A. L., Rich, R. R., David, J. R., and Pierce, C. W., J. Immunol. **117,** 323 (1976).

Received September 7, 1976. P.S.E.B.M. 1977, Vol. 154.