Effect of Phytohemagglutinin on Hemolytic Plaque Formation by Rat Lymphoid Cells* (32731)

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Phytohemagglutinin (PHA) has been reported to depress or to enhance the immune response, depending on a variety of different experimental variables (1–7). This paper presents observations in rats on the effect of PHA *in vitro* and *in vivo*. Under different circumstances the mitogen was found to depress or stimulate hemolytic plaque formation.

Materials and Methods. Cells of thoracic duct lymph (TD cells), spleen, and thymus were harvested from male Lewis rats. They were washed once, counted, and adjusted to the desired concentration by addition of Earle's balanced salt solution (BSS)[2]. One $\times$ 10^7 cells, in 1 ml, were incubated at 37°C in plastic petri dishes, 35 mm in diameter; they were cultured over a period of 96 hours in an atmosphere of 90% air and 10% CO_2.[3] Cells were removed from the culture dishes by flushing with the aid of a Pasteur pipette containing MEM. Survival was calculated by counting intact nucleated cells in a hemocytometer after dilution in a leukocyte pipette with 1% acetic acid.

Hemolytic plaque forming cells (PFC) were assayed by a modification of Jerne's technique (8) using regular microscope slides and agarose in MEM without bicarbonate. Up to 3 $\times$ 10^6 cells were plated on each slide and results were expressed as PFC/10^6 cultured cells which were recovered from the incubation.

Phytohemagglutinin (PHA-M or PHA-P)[4] was dissolved in 25 ml of BSS for *in vitro* studies and in 5 ml of phosphate buffered saline, pH7 (PBS 7), for *in vivo* experiments. Nitrogen content was determined by micro-Kjeldahl analysis. Mitogenic activity was assayed by calculating the percentage of labeled cells in autoradiographs of brush smears prepared from cultures of cells which had been incubated for 96 hours in the presence of 3.6 μg N/ml of PHA-M and thymidine-^3H[5], 1.5–2 μC of the latter being added daily.

Results. Survival of TD, splenic, and thymic cells after 96 hours of incubation, was 50%, 25%, and 10%, and the number of PFC ranged from 4–60, 4–37, and 6–46, respectively. Without prior *in vitro* incubation, these 3 cell populations consistently yielded fewer than 1 PFC. The increase of hemolytic plaques, after 96 hours of culture, was due to the presence of FBS, as was reported for mouse cells by Mishell and Dutton (9). If FBS was replaced by normal bovine serum,[2] by microgamma calf serum,[2] or by a 1:1 mixture of an ultrafiltrate of bovine serum and dialyzed rat serum, the number of PFC which was found at 96 hours of incubation was less than 0.1/10^6, with cell survival being equally as good as when FBS was present in the medium. The FBS was therefore added to all culture medium in the following experiments:

1. The effect of PHA-M on the number of PFC in vitro. The effect of PHA-M on the number of PFC was studied in cultures of cells taken from rats with or without prior exposure to sheep red cells. Either 18 or 3.6 μg

* Publication No. 252 from Dept. of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif. This study was supported by U. S. Public Health Service Grant AI-7007.

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[3] The culture medium consisted of: Eagle's minimum essential medium (MEM); L-glutamine, 0.004 *M*; sodium pyruvate, 0.001 *M*; nonessential amino acids; final concentration of dextrose, 0.022 *M*; penicillin, 100 u/ml; streptomycin, 100 μg/ml; 20% fetal bovine serum (FBS); 0.15 ml of a nutritional mixture (9). All components except the last were obtained from Microbiological Associates.

[4] Difco Laboratories, Detroit, Mich.

[5] Schwarz Bio-Research, Orangeburg, N. Y.; specific activity, 6C/mmole.

TABLE I. Effect of PHA-M on the Number of PFC in Culture.

Each row represents one experiment in which 15×10^7 cells (in 15 culture dishes) were incubated with PHA-M and 15×10^7 cells of the same pool without PHA-M. After 96 hours of incubation the cultures were pooled and assayed for PFC by the Jerne technique.

Type of cell	Time in culture (hours)	Concentration of PHA-M (μg N/ml)	PFC/10^6 with PHA-M	PFC/10^6 without PHA-M
TD	96	18.0	0.13	14.40
TD	96	18.0	0.48	29.10
TD	96	3.6	5.23	29.10
Spleen	96	18.0	0	13.50
Spleen	96	3.6	0.52	13.50
Spleen	96	3.6	2.70	36.70
Spleen	96	3.6	0	18.00
Spleen	96	3.6	0	3.60
Thymus	96	3.6	0.78	5.79

N of PHA-M in 0.05 ml of BSS were added to each ml of medium containing 1×10^7 nucleated cells; control cultures without PHA-M were incubated simultaneously. As shown in Table I, the mitogen reduced the number of PFC in every instance. When incubated for 96 hours in medium containing thymidine-^{3}H and 3.6 μg N of PHA-M, 15% of TD lymphocytes were found to be labeled.

In another set of experiments, PHA-M was added from 0–3 days after the cultures were incubated. Control preparations, without PHA-M, yielded an average of 37 PFC; cultures exposed to PHA for 4 days yielded 3 PFC; for 3 days, 5 PFC; for 2 days, 18 PFC; and for 1 day, 34 PFC.

2. The effect of PHA-P on the number of PFC in vivo. The 750 μg N of PHA-P (which is 50 $\times$ more potent than PHA-M) was injected into groups of 3 rats, ranging between 140–160 gm, before, at the time of, and after the administration of 1 ml of a 25% suspension of SRC. The number of PFC obtained from the spleens was considerably reduced if the PHA was infused beginning 3 or 2 days before antigen injection or 2 or 3 days after (Table II). By contrast, the number of PFC was approximately doubled when PHA-P was administered from 1 day before to 1 day after the injection of SRC.

Discussion. The number of PFC in rat lymphoid tissues (spleen and thymus) or in TD cells, taken directly from the host without *in vitro* incubation was less than 1 per 10^6 plated cells. These "background" PFC were increased markedly, up to 60 per 10^6 plated cells, when lymphoid cells were maintained for 4 days with FBS in the tissue culture medium. The nature of the cells producing "background" plaques has not been satisfactorily elucidated. These cells may be considered as precursor elements, i.e., a cell type which has not been previously exposed to antigen and which can divide and differentiate into an antibody producing cell when stimulated by antigen. They may also be regarded

TABLE II. Effect of PHA-P *in Vivo* on the Number of PFC in Spleens of SRC Immunized Rats.

The SRC injection was given on day 0. Numbers preceded by a — sign represent days before, while numbers preceded by a + sign, days after immunization. The Jerne assay was performed 4 days following antigen injection. The number of PFC/10^6 spleen cells represents the mean calculated from 3 pools of 3 spleens each.

PHA-P given (days) •	Number of PFC/10^6 spleen cells ($\pm$ SE of the mean)	Percentage of control PFC (%)
—3, —2, —1	133 $\pm$ 31	24
—2, —1	360 $\pm$ 47	66
—1	1093 $\pm$ 90	199
0	1058 $\pm$ 52	193
+1, +2, +3	1190 $\pm$ 80	217
+2, +3	288 $\pm$ 14	52
+3	400 $\pm$144	73
No PHA-P	548 $\pm$ 74	100

as residual sensitized elements prevalent in lymphoid tissues which have met previously with sheep or other antigens similar to or identical with antigens of SRC. If the latter is true, the increase of "background" cells in the presence of FBS could be interpreted as an anamnestic response to antigen which is related to SRC. In studies not recorded here, sera from rats immunized with SRC were tested by immunodiffusion in Ouchterlony plates and by immunoelectrophoresis against FBS. No precipitin lines developed. Furthermore, extracts of soluble antigens from SRC did not cross-react with antisera to FBS. The other alternative would ascribe the increase of "background" PFC to a boosting of proliferative activity of all precursor cells by FBS, among which class were those capable of developing into cells that could synthesize antibody against SRC.

Whatever the "background" PFC were, their numbers were decreased *in vitro* by PHA-M in the medium. The PHA was used at concentrations which were not overtly toxic, since the survival rates of incubated cells with and without this reagent were similar, and also since mitotic activity was high in cultures with PHA-M. The decrease of "background" PFC, therefore, could not be attributed to a depressing effect on cell division. We might speculate that the cells which eventually synthesize antibody to SRC were prevented from differentiating into this role because of their intense mitotic activity.

In vivo our results indicated that PHA-P decreased the number of PFC when given more than 1 day before or after antigen, while it increased the number of PFC when administered shortly before or after the injection of antigen. The series of events which occur *in vivo* following the use of PHA-P and antigen are too complex to permit an understanding of what is occurring at the cellular level. Indeed, our data merely add to the number of reports which demonstrate in several species that PHA may enhance the immune response or depress it, depending upon a variety of variables.

Summary. Rat TD, splenic, and thymic cells, with or without prior exposure to SRC *in vivo,* yielded reduced numbers of PFC when the lymphoid cells were incubated for 96 hours in a medium containing PHA-M during 1–4 days of the incubation. The infusion of PHA-P into rats 3 and 2 days before and after the intravenous injection of SRC resulted in a diminution of PFC in the spleen. The administration of PHA-P to rats 1 day before, at the time of, or 1 day after antigen injection resulted in an increase of PFC in the spleen.

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Received Oct. 12, 1967. P.S.E.B.M., 1968, Vol. 127.