## Effect of Trypsin on Phytohemagglutinin Stimulated DNA Synthesis in Rat Spleen Cell Cultures

Extensive studies have been conducted on the mitogenic properties of phytohemagglutinin (PHA) and pokeweed mitogen (PWM). To a large extent these studies have been confined to peripheral blood lymphocytes isolated from human subjects. A few studies have been conducted on rabbit spleen[1] and rabbit peripheral blood lymphocytes[2] and pig thymic cells[3,4]. To the best of our knowledge there have been no reports dealing with mitogen stimulation of rat and mouse lymphocytic cells.

Present studies are concerned principally with the effects of PHA on the stimulation of tritiated thymidine incorporation into DNA of rat spleen cells ($H^3$-TdR → DNA) in short-term cultures. By trypsinization of dispersed spleen cells prior to the addition of PHA, it has been possible, in consecutive experiments to routinely stimulate DNA synthesis in short term cultures of spleen cells. The smaller variations in these laboratory animals as well as the increased cellularity of spleen tissue should experimentally facilitate studies dealing with early cellular and biochemical mechanisms associated with PHA stimulation.

*Materials and methods.* Male rats weighing 150–200 g were obtained from Simonsen Laboratory, Inc., White Bear, Minnesota. Concentrates of essential amino acids, vitamins, penicillin-streptomycin and L-glutamine, and fetal calf serum were obtained from GIBCO, Grand Island, New York. 16 × 150 mm flint glass tubes were obtained from Bellco Glass Co., Vineland, New Jersey. Trypsin was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. Phytohemagglutinin-P (PHA-P) was obtained from Difco Laboratory, Detroit, Michigan. $H^3$-TdR, sp. act. 0.36 c/m*M*, was obtained from Schwartz Bioresearch, Inc., Orangeburg, New York.

Preparation of cell suspension. Spleens were removed aseptically and placed in modified Hank's salt solution (HSS) pH 7.4[5]. All manipulations were performed at room temperature. The cells were gently teased and passed through a 50 mesh stainless steel wire screen and collected by centrifugation at 800 rpm for 15 min in a PR-2 International Refrigerated Centrifuge (head No. 269). The supernatant fluid was removed and the cell pellet was resuspended in HSS by gently mixing with a Vortex mixer. The cells were washed twice with HSS, electronically counted with a Coulter counter model B, and diluted to $1.5 \times 10^7$ cells/ml of HSS containing EAGLE's nutrients[6] and 8% fetal calf serum. Two ml of cell suspension (containing $3 \times 10^7$ cells) were transferred to 16 × 150 mm flint glass culture tubes and were capped with silicon rubber stoppers. They were incubated at a 5 degree slope in a 37 °C incubator using air as the gas phase.

Prior trypsinization of dispersed spleen cells were accomplished in the following manner: 2 mg of trypsin contained in 0.2 ml of HSS were added to each 2 ml of cell suspension in HSS and incubated at 37 °C for 30 min. The cells were washed twice and suspended in the complete media (specified above) for short-term culture.

Preliminary experiments indicated that PHA stimulation of DNA synthesis was optimal when added immediately before incubation.

Determination of $H^3$-TdR incorporation into DNA (acid-insoluble precipitate). The proliferative response to PHA was measured by the incorporation of $H^3$-TdR into acid-insoluble precipitate of cultured spleen cells. Two $\mu$C of $H^3$-TdR was added per 2 ml culture at 20 h and the cells were harvested at 44 h. The cells were washed once with ice cold saline, twice in ice cold 5% trichloracetic acid and twice in ice cold methanol[7]. The washed cells were digested in 0.2 ml formic acid in an 80 °C oven overnight and the radioactivity determined by liquid scintillation counting using BRUNO and CHRISTIAN's counting media[8].

*Results.* Preliminary experiments indicated that the cellular proliferative response to PHA measured by incorporation of $H^3$-TdR into dispersed rat spleen cultures was erratic; spleen cells were stimulated or inhibited by PHA. This is clearly shown on the Table and Figure 1 (non-trypsinized spleen cells). Experiments 1, 3, 15 and 16 (Table) showed that PHA addition was inhibitory to thymidine incorporation and that this inhibition by PHA was dose dependent; the ratio of (+ PHA/− PHA) $H^3$-TdR incorporation was decreased with the addition of higher concentrations of PHA. Also experiments 2, 7 and 10 demonstrated that the degree of stimulation did not always correspond with PHA doses. Figure 1 summarizes the response of non-trypsinized spleen cells to PHA addition. The addition of 5 $\mu$g of PHA/ml cultures brought about an inhibitory or an insignificant effect in 3 out of 5 experiments. Similarly, 3 out of 6 experiments at 50 $\mu$g of PHA/ml culture and 2 out of 6 experiments at 500 $\mu$g of PHA/ml cultures were either inhibitory or not significantly different from the controls.

On the other hand, pre-trypsinization of dispersed spleen cultures resulted in a consistent proliferative response to PHA (Figure 2). In addition, the values shown in the Table (trypsinized spleen cells) indicate that, with increasing doses of PHA, there was an increase in the amount of $H^3$-TdR incorporation. Thus, except for one observa-

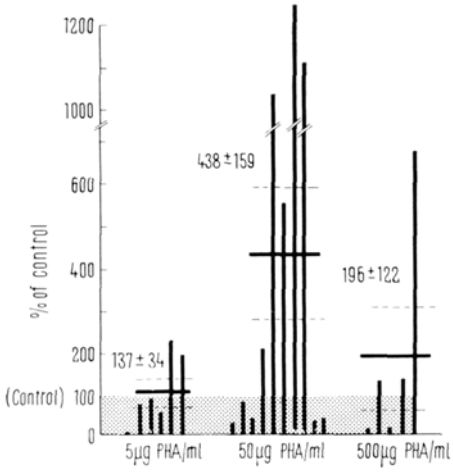


Fig. 1. $H^3$-TdR → DNA of short-term spleen cell culture (non-trypsinized). Each bar represents 1 experiment. The bars in shaded area represent cultures inhibited by PHA at various dose levels. Numbers at each dose level represent mean ± standard error of the mean.

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tion (Table, experiment 4 at 5 $\mu$g PHA/ml culture) all pre-trypsinized spleen cultures were stimulated to incorporate $H^3$-TdR. The degree of stimulation was proportional to PHA at 5 $\mu$g/ml and at 50 $\mu$g/ml dose levels. It may also be noted that although the degree of $H^3$-TdR incorporation in trypsinized and in non-trypsinized cultures was comparable percentage-wise, at 50 $\mu$g PHA/ml (i.e., 438 vs 418% control), the actual incorporation of $H^3$-TdR into pre-trypsinized spleen cultures was higher in all experiments. Thus, a comparison of experiments in which trypsinized and non-trypsinized cultures were run simultaneously (Table, experiments 7, 10, 12, 13 and 14) showed that the average incorporation of $H^3$-TdR was 2960 and 1290 dpm for 2 ml of culture for trypsinized and non-typsinized cultures, respectively.

Experimental studies dealing with varying concentrations of trypsin indicated that, although wide variations were noted from animal to animal, trypsin levels in the range of 0.5–1.0 mg/2 ml of spleen cells gave optimal levels of $H^3$-TdR incorporation; higher concentrations of trypsin (2.0–4.0 mg/2 ml culture) gave values that were generally lower although still above control (without added PHA) values.

It has also been our observation that other rodent short-term spleen cultures (mouse, hamster and guinea-pig) are similarly stimulated by pre-trypsinization.

*Discussion.* The present experiments involving pre-trypsinization of short-term cultures of dispersed rat spleen cells clearly indicated a consistent and reproducible stimulation of $H^3$-TdR incorporation. Without prior trypsinization, the incorporation of $H^3$-TdR was not consistent (i.e. stimulation or inhibition). The present study, therefore, offers a methodological advantage in the further analysis of morphological and biochemical responses to PHA stimulation in common laboratory animals, utilizing splenic tissues which are of sufficient cellularity (ca. $7 \times 10^8$ nucleated spleen cells) to run 20–30 cultures from 1 animal.

The wide variations in $H^3$-TdR incorporation in some control tubes from different animals is noteworthy. A

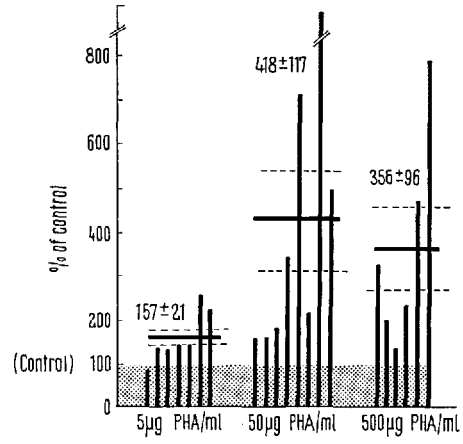


Fig. 2. $H^3$-TdR → DNA of short-term spleen culture (trypsinized). Only 1 bar (5 $\mu$g dose) is in shaded area. For details see text. Numbers at each dose level represent mean ± standard error of the mean.

Tritiated thymidine incorporation into DNA of short-term spleen culture $H^3$-TdR → DNA (in dpm/2 ml containing $3.0 \times 10^7$ spleen cells)

| PHA doses/ 2 ml culture | Experiment No.: 1 | 2 | 3 | 7 | 10 | 12 | 13 | 14 | 15 | 16 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Non-trypsinized spleen cells | | | | | | | | | | | |
| 0 | 1140 | 150 | 1150 | 320 | 820 | 510 | 400 | 4400 | 445 | 310 | |
| 10 $\mu$g | 1010 | 150 | 970 | 760 | 1780 | – | – | – | – | – | |
| 100 $\mu$g | 430 | 130 | 685 | 620 | 9010 | 2880 | 4970 | 48600 | 138 | 133 | |
| 1000 $\mu$g | 140 | 206 | 180 | 490 | 5830 | – | – | – | – | – | |
| Ratio of $H^3$-TdR → DNA (with PHA)/$H^3$-TdR → DNA (without PHA) | | | | | | | | | | | |
| 0[a] | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | Average[b] |
| 10 $\mu$g | 0.87 | 1.00 | 0.61 | 2.37 | 2.04 | – | – | – | – | – | 1.37 ± 0.34 (5) |
| 100 $\mu$g | 0.36 | 0.87 | 0.44 | 2.00 | 10.35 | 5.64 | 12.42 | 11.04 | 0.31 | 0.43 | 4.38 ± 1.59 (10) |
| 1000 $\mu$g | 0.12 | 1.37 | 0.11 | 1.53 | 6.70 | – | – | – | – | – | 1.96 ± 1.22 (5) |

| PHA doses/ 2 ml culture | Experiment No.: 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Trypsinized spleen cells | | | | | | | | | | | | | | |
| 0 | 3100 | 7430 | 850 | 550 | 340 | 600 | 780 | 210 | 1137 | 1740 | 10590 | – | – | |
| 10 $\mu$g | 2860 | 9790 | 1130 | 770 | 470 | 1520 | 1710 | – | – | – | – | – | – | |
| 100 $\mu$g | 4860 | 9530 | – | – | 600 | 2340 | – | 1500 | 2380 | 18660 | 53230 | 39900 | 29250 | |
| 1000 $\mu$g | 9980 | 14960 | 1170 | – | 810 | 2780 | 6110 | – | – | – | – | – | – | |
| Ratio of $H^3$-TdR → DNA (with PHA)/$H^3$-TdR → DNA (without PHA) | | | | | | | | | | | | | | |
| 0[a] | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | | | Average[b] |
| 10 $\mu$g | 0.92 | 1.31 | 1.32 | 1.40 | 1.37 | 2.53 | 2.20 | – | – | – | – | | | 1.57 ± 0.21 (7) |
| 100 $\mu$g | 1.56 | 1.28 | – | – | 1.75 | 3.90 | – | 7.14 | 2.10 | 10.72 | 5.02 | | | 4.18 ± 1.17 (8) |
| 1000 $\mu$g | 3.21 | 2.01 | 1.37 | – | 2.34 | 4.64 | 7.83 | – | – | – | – | | | 3.56 ± 0.96 (6) |

[a] 0 PHA values are considered as unity. [b] Values represent the mean ± standard error of the mean. The number of determinations used for each value is in parentheses.

number of possible explanations which have not yet been experimentally considered are the following: (1) variability in the amount of 'cold' or endogenous TdR pool to dilute the labeled thymidine, (2) variability in the proportion of spleen cells capable of undergoing mitosis (and therefore $H^3$-TdR → DNA) without PHA stimulation, and (3) variability of cells to respond to fetal calf serum as an antigen.

The mechanisms by which trypsin enhances $H^3$-TdR incorporation into spleen cell cultures are not known. A recent observation[9] on human peripheral blood lymphocytes indicating that trypsinization acts to inhibit thymidine incorporation would appear to be inconsistent with our present findings. However, our studies indicated that higher than optimal concentrations of trypsin lowered the ability of spleen cultures to respond to PHA. Studies have shown that PHA or PWM[10,11] stimulated lymphocytes possessed fine structural alterations as compared with non-PHA treated lymphocytes; in particular, these studies have indicated that there was an increase in vacuoles containing acid phosphatase, an enzyme which is closely associated with lysosomes. These studies, together with our studies on trypsin action on isolated spleen cells, are consistent with the notion that a certain degree of injury, perhaps, to releasing lysosomal enzymes, may be important in the induction of blastogenesis. HIRSCHHORN and HIRSCHHORN[12] suggested that rupture of a few lysosomes of a small lymphocyte may be an early stimulus to derepression of this cell type[13].

*Résumé.* On a étudié l'action de la phytohémagglutinine (PHA) sur la stimulation de l'incorporation de thymidine tritiée à la DNA de cellules spléniques de rats, en cultures à court terme. Il a été possible de stimuler la synthèse de la DNA dans ce type de cultures au moyen de la trypsinisation des cellules dispersées avant l'addition de la PHA. Les variations relativement faibles observées dans ce type d'essai vont faciliter les études des mécanismes cellulaires et biochimiques associés à la stimulation par la PHA.

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## The Turbidimetric Evaluation of Platelet Aggregation Caused by Bacterial Lipopolysaccharide

Aggregation of blood platelets occurs in the presence of adenosine diphosphate (ADP), thrombin, collagen, fatty acids, antigen-antibody complexes, and bacterial lipopolysaccharide (endotoxin). ADP causes aggregation initiated by several of these agents[1,2]. The dynamics of aggregation and the effects of inhibitory substances have been determined for many aggregating materials using a turbidimetric technique[3]. That technique, however, does not appear to have been used to characterize aggregation by bacterial lipopolysaccharide (LPS). The results of such a study are described in this report.

Sprague Dawley rats weighing 350 g were anesthetized with diethyl ether by inhalation. Aortic blood obtained through polyethylene cannulae was collected in plastic (Nalgene[4]) tubes into $^1/_{10}$ volume of heparin sodium[5] in imidazole buffered (pH 7.4) sodium chloride solution (0.154 *M*). Unless otherwise indicated, heparin was diluted ($^1/_{100}$) in buffer, the final heparin concentration being 1 U/ml of whole blood. Blood was centrifuged for 7 min at 175 *g* (4 °C) to obtain platelet rich plasma (PRP, approximately 600,000 platelets/mm³). PRP was stored at 10–15 °C until used. When necessary, PRP was diluted with platelet poor plasma. The study of aggregation was completed 30 min after collection of blood. All experiments were paired; 2 samples of PRP were used from each rat, one being a suitable control, the other an experimental sample. In studies using varying amounts of heparin, blood was collected in separate tubes. Platelet aggregation was studied turbidimetrically at 37 °C with a Bausch and Lomb spectrophotometer as described[6] although optical density changes were determined at 620 nm. *Escherichia coli* LPS (control 0127:B8) was used in all studies. LPS was dissolved in imidazole buffered saline (concentration of 0.5 mg/ml), and 0.1 ml was added to 2.0 ml of PRP. Before addition of LPS, all samples were warmed at 37 °C for 2 min, and the optical density was determined at 1 min intervals for 3 min. Following addition of LPS or IBS, samples were stirred continuously for 5 min, with O.D. being recorded at 15 sec intervals. Thereafter, O.D. was recorded every min for 5 min, and the experiment terminated.

Addition of LPS to PRP caused an initial slow decrease followed by an accelerated phase of decrease in O.D. The onset of the accelerated decrease was as early as 75 sec and as as late as 270 sec after LPS. The slower phase of decrease generally occurred during the first 10% of the fall in O.D. Mean total changes in O.D. in control and experimental samples during the 10 min period were 5% and 61% respectively (Figure 1). In 10 rats the final heparin concentration was 10 u/ml, and 1 u/ml in another 10 rats. The mean initial O.D. of PRP in the former group was 0.70, and 0.68 in the latter group. Using 10 u/ml the accelerated decrease of O.D. was retarded (Figure 1), but until 135 sec after LPS mean differences in O.D. in the 2 groups were $\leqslant 6\%$. Statistically significant differences in mean values were observed 4 min after addition of LPS

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