Studies on the Blastogenic Response of Murine Lymphocytes II. The Effects of Serum–Stimulant–Cell Interactions on Phytohemagglutinin-Induced Stimulation (36393)

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In a previous report (1) we have described some of the parameters affecting the blastogenic response of murine lymphocytes on stimulation with phytohemagglutinin (PHA) and noted that the degree of transformation was dependent on the cell density and the serum level in the culture medium as well as the PHA concentration.

In the present investigation, the interactions between these three factors which affected the blastogenic process were examined. It had been demonstrated in several studies of the culture system that it was necessary to adjust the concentration of PHA relative to the concentration of serum in the medium in order to achieve maximum blastogenesis (2–4). In other studies, the cell density also had been found to influence the blastogenic response (5–7). However, a dependency on the cell density of either the PHA concentration or the serum concentration similar to that between PHA and serum has not been studied directly.

In order to clarify the nature of the serum effect, albumin, gelatin and methylcellulose were used as substitutes for whole serum.

Methods and Materials. Cells and culture conditions. Pooled spleen lymphocytes obtained from male NIH BALB/c AnN mice were processed and cultured as described previously (1), with the exception that the cells were incubated in 12 × 35 mm (1/2 dram) glass flat-bottom vials (Kimble) capped with loose-fitting autoclavable closures ("Bacti Capall," Biological Research).

Assay of nucleoside incorporation. After incubation for 24 hr at 37°, the cell cultures were pulsed for a period of 18 hr with 0.5 μCi of ^{3}H-thymidine (1.9 Ci/mmoles, Schwarz/Mann). Incorporation of labeled nucleoside was assayed by a slightly modified version of the procedure described by Ling (8). All procedures were performed directly in the culture vessels. The simultaneous washing of up to 28 cultures was accomplished by centrifugation in a multiplace carrier (International Equipment Company No. 1021) followed by decantation through a wire screen. The acid-insoluble fraction obtained by precipitation with 5% trichloroacetic acid at 4° was dissolved in 0.2 ml of "NCS Solubilizer" (Amersham/Searle) prior to blending with 10 ml of scintillation fluid ("Liquifluor", New England Nuclear).

The 1/2 dram vials containing the alkaline solutions of labeled products were placed directly into the standard bottles used for scintillation counting, thus avoiding the need for the transfer of samples.

Reagents and suppliers. Purified phytohemagglutinin (MR69) (Burroughs-Wellcome, Tuckahoe, NY); fetal calf serum (FCS) (Grand Island Biological Company, Grand Island, NY); crystallized bovine serum albumin (BSA) (Pentex Division, Miles Laboratories, Kankakee, IL); gelatin (Nutritional Biochemicals, Cleveland, OH); methylcellulose (Methocel, 15 cps grade) (Dow Chemical Company, Midland, MI).

Analysis. The total protein (4.0%) and the albumin content (2.5%) of the FCS were determined by a biuret method (9). For the purposes of comparison, a solution of BSA at a concentration of 1 mg/ml was considered to be equivalent to a 4% solution of FCS, on the basis of albumin content.

Results. Effect of variation of cell density, stimulant concentration and supplement concentration, with two different supplements. Under customary experimental conditions, where all factors but one were present in fixed amounts, the three components of the

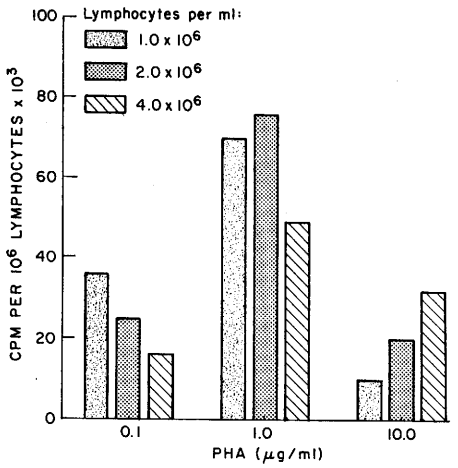

FIG. 1. Effect of variation in cell density on thymidine incorporation in PHA-stimulated lymphocyte cultures over a series of PHA concentrations. The concentration of serum was 4%. The plotted values of the histograms were the averages of the counts obtained from replicate cultures.

blastogenesis system under investigation (lymphocytes, PHA, serum) appeared to be functioning as independent parameters in that the extent of thymidine incorporation was dependent upon the concentration of the variable factor (1). In order to detect the effects of possible interactions between the components of the blastogenesis reaction mixture it was found necessary to vary two parameters concurrently. Such an experiment is illustrated in Fig. 1, where the thymidine uptake of mouse spleen lymphocytes exposed to a series of PHA concentrations was measured at three different cell densities. It was evident that cell density had a marked effect on the stimulatory activity of PHA. At a suboptimal concentration of PHA (0.1 μg/ml), an increase in cell density from 1.0 $\times$ 10^6 to 4.0 $\times$ 10^6 cells/ml produced a significant decrease (50%) in the specific activity of the PHA. On the other hand, the same increase in cell density in the presence of an inhibitory concentration of PHA (10 μg/ml) caused a threefold increase in the thymidine uptake/cell, which resulted in a diminution of the suppressive effect of the PHA.

The bivariant system just described yielded highly suggestive data but was too limited in scope to provide adequate information on all the interactions involved. To gain such information, "checker-board" type experiments were performed where a series of concentrations of stimulant were varied against a series of concentrations of supplement in the presence of two different concentrations of cells. In order to clarify the results, dose–response curves were transformed to a common basis by normalizing each curve against the maximum count for thymidine uptake obtained with that particular system. This procedure yielded the families of curves shown in Fig. 2, which are based on PHA as the dose-dependent variable.

On examination of the dose–response curves obtained with serum as the supplement at a cell density of 1 $\times$ 10^6 cells/ml (Fig. 2a) and of 2 $\times$ 10^6 cells/ml (Fig. 2b), a number of relationships became apparent. For example, it was evident that, as the concentration of FCS was increased, the concentration of PHA required to yield the maximum response attainable with that particular serum concentration also increased. It may be noted that the reciprocal relationship also held, namely, that, as the concentration of PHA was increased, the concentration of serum required to support maximum blastogenesis also increased.

A consequence of this interdependency between PHA and FCS can be observed by comparison of the PHA dose–response curves for two different concentrations of serum, one relatively low and the other relatively high. The effects were particularly evident on the apparent reactivity of suboptimal concentrations of PHA. Thus, for example, for a cell density of 2 $\times$ 10^6 cells/ml (Fig. 2b), the optimal concentration of PHA in the presence of 2% FCS was 1 μg/ml, with 0.2 μg/ml of PHA yielding a response about 65% of maximum. When the serum supplement was increased to a level of 20%, the amount of PHA required to produce maximum thymidine incorporation was increased to a concentration of 4 μg/ml. More striking, the suboptimal concentration of PHA of 0.2 μg/ml of PHA, which displayed significant activity in the presence of a low concentration of serum, was now no longer effective in

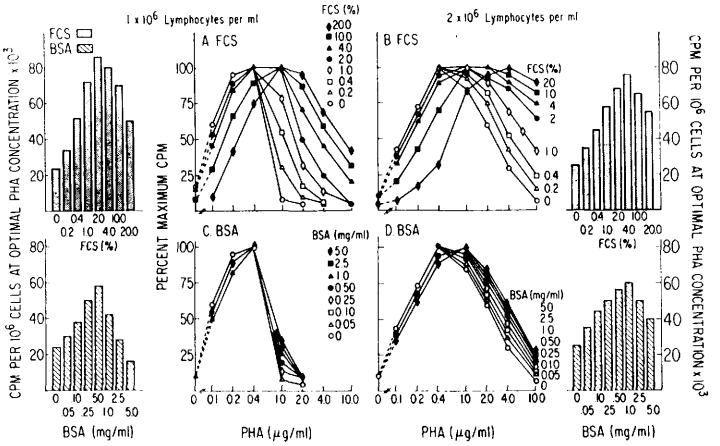

FIG. 2. Effect of variation in concentration of supplemental FCS and BSA over a range of PHA concentrations, each compared at two different cell concentrations. The counts obtained with each concentration of supplement at the optimal concentration of PHA are shown in the histogram plots adjacent to the respective dose–response curves. The two sets of histograms are arranged so that, on vertical comparison, BSA concentrations (mg/ml) are equivalent, on the basis of albumin content, to the corresponding values for FCS (vol % of culture fluid). The data plotted as histograms and as point values were derived as described in the legend of Fig. 1.

the "high-serum" medium.

On comparison of the respective curves of Fig. 2b with those of a, it was evident that, as the cell density was increased, the apparent reactivity of a fixed suboptimal concentration of PHA was decreased, as already shown by the data in Fig. 1. However, as demonstrated in Fig. 2, the response was restored with the higher cell density when the amount of PHA in the medium was increased. In addition, examination of the histogram plots shown in Fig. 2a and b indicated that a higher serum concentration was required to achieve maximum thymidine uptake with the higher cell density.

Blastogenesis in the absence of supplement. As negative controls for the series of experiments illustrated in Fig. 2, reaction mixtures containing no added supplement were included. It was evident from the histogram plots in Fig. 2a and b that significant blastogenesis occurred in the absence of added supplement (about one-third of the maximum response).

Serum albumin as a substitute for whole serum. In Fig. 2c and d are illustrated the data obtained when whole FCS was replaced by BSA, the principal protein constituent of FCS. The figures are arranged so that comparisons can be made on an equivalent basis both vertically and horizontally. BSA was about two-thirds as effective as whole serum in enhancing blastogenesis as measured by the increase in thymidine incorporation. Comparison of the several sets of curves indicated that the simple protein behaved similarly to whole serum in almost all respects, including the effect of an increased supplement requirement to achieve maximum response with an increased cell density. However, it was evident from Fig. 2, that BSA was different from FCS in at least one kind of interaction since the range of concentrations of PHA yielding optimal transformation was markedly narrower (0.4 to 1.0 μg/ml) than observed with FCS (0.4 to 4.0 μg/ml).

Gelatin and methylcellulose as replacements for BSA. The effects of gelatin and of methylcellulose as supplements to the culture medium are illustrated in Fig. 3 under conditions identical to those for the data in Fig. 2d. Comparison of the two figures shows that both gelatin and methylcellulose were equivalent to BSA in enhancing blastogenesis. Methylcellulose was less effective, however, than the other materials at high concentration (5 mg/ml), presumably because of its limited solubility. Variation in the concentra-

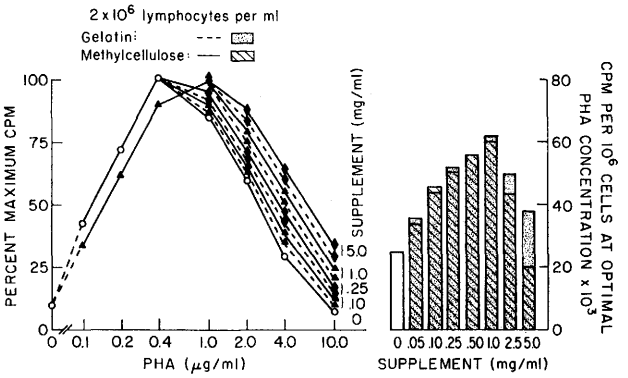

FIG. 3. Effect of gelatin and of methylcellulose in supporting blastogenesis over a range of PHA concentrations.

tion of gelatin and of methylcellulose, like BSA, showed little effect on the relationships between the PHA dose–response curves.

Discussion. The results demonstrated that while the blastogenic process was dependent on the concentration of stimulant, the density of cells, and the amount of serum added to the culture medium, the specific response obtained with any combination of these three parameters was governed by the relative concentrations of these three factors. Not only the relative concentrations of PHA and supplement but also that of cells and PHA and that of cells and supplement controlled the blastogenic process.

One explanation of this interdependency is that the three factors were interacting with each other to modify the amount of each factor available for the stimulatory process. The reactions involved are illustrated in Fig. 4. In support of this hypothesis are the many observations that PHA is bound both by lymphocytes and by serum. Binding of PHA by cells (Fig. 4, Reaction A) has been shown indirectly by the effect on the biologic activity of the PHA (10) and directly with the aid of labelled material (11). Binding of PHA by several components of normal serum (Fig. 4, Reaction B) has been established by means of the gel precipitation technique (12, 13). The third reaction, that of supplement and cells, (Fig. 4, Reaction C) has not been demonstrated directly but has been suggested as a possibility in explanation of the effect of serum in blastogenesis (2, 3).

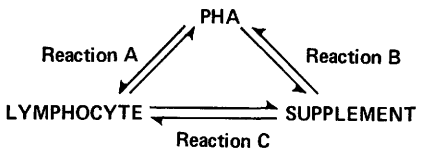

FIG. 4. Interactions of three components of the blastogenesis system: lymphocyte-PHA-supplement.

The triad of equilibria depicted in Fig. 4 can account for all the experimental results. The observations that thymidine uptake was dependent on the concentration of the variable factor when the other parameters were fixed but was dependent on the relative concentrations of two factors, e.g., PHA - serum ratio, when the two factors were varied, are in agreement with this concept. Further, the two reactions involving PHA represent a reversible system which can account for the findings that an increase in the concentration of either cells or serum resulted in a reduction of the stimulatory effect of a low concentration of PHA and in a lessening of the suppressive effect of a high concentration of PHA.

The reactions shown in Fig. 4 are useful in understanding some of the properties of the three types of culture media available for supporting the blastogenic process. Type I may be defined as a culture medium containing no added supplement and, hence, only Reaction A (Fig. 4) between PHA and cells can occur. Type II is a culture medium containing a supplement which combines with the cell but not with the stimulant, where both Reaction's A and C (Fig. 4) take place. Finally, Type III is a culture medium containing a supplement which combines with

both the cell and the stimulant and where all three reactions shown in Fig. 4 take place.

The appreciable thymidine uptake occurring in the absence of any supplement (Type I), had been observed previously (14). This may be a special characteristic of the murine spleen lymphocyte system since lymphocytes from only a few other species have been stimulated in a completely synthetic medium, e.g., chicken (15).

Lymphocytes of human origin have been successfully stimulated in media supplemented with dextran (16), serum albumin (17), methylcellulose (18), or a modified gelatin (19). In our experiments where either BSA, gelatin or methylcellulose was used as a supplement, the convergence of the sets of PHA dose–response curves (Figs. 2 and 3) indicated that none of these supplements appeared to react significantly with PHA (Type II). The lack of interaction with PHA displayed by serum albumin had been noted in the serum-binding studies previously mentioned (12, 13). This was surprising in view of the affinity of albumin for a variety of molecular species (20). That the three supplements did combine with murine spleen lymphocytes (Fig. 4, Reaction C) was suggested by the fact that an increase in cell density required a corresponding increase in the concentration of supplement, if the original level of thymidine uptake were to be maintained. The putative interactions with the lymphocyte appeared to be nonspecific in nature since the three substances enhanced the blastogenic response to the same extent despite their chemical dissimilarity. It may be relevant that all three materials have been used in tissue culture media as "protective agents" for cells (21).

Types I and II culture media have the advantage of being chemically defined. They have the disadvantage of not supporting the rate of DNA synthesis attainable with a serum-containing medium. Further, cells in such media are very sensitive to the toxic effects of PHA above a sharply defined optimal concentration. Whole serum in the Type III culture medium contributes, in addition to its stimulatory effect, a buffering effect so that the inhibitory effects of excessive concentrations of PHA are minimized, permitting the use of PHA over a wider range of concentrations. The disadvantage of the use of whole serum is the introduction of a number of extraneous and variable factors.

Summary. In PHA-induced blastogenesis of mouse spleen lymphocytes cultured in a medium supplemented with serum, the specific response obtained with any combination of these three variables was governed by their relative concentrations, indicating that the three factors interacted with each other.

The PHA-binding function of serum exerted a buffering effect which was expressed as a suppression of the activity of very low concentrations of PHA and as a tolerance of levels of PHA which otherwise would have been inhibitory. The PHA-binding caapcity of lymphocytes resulted in a similar effect.

A medium containing no supplement supported a moderate degree of blastogenesis. Supplementation with either serum albumin, gelatin or methylcellulose provided a chemically defined medium which supported blastogenesis nearly as well as with whole serum. However, cells in such media were very sensitive to the toxic effects of PHA in concentrations above a sharply defined maximum.

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