SYNERGISTIC AND SUPPRESSIVE INTERACTIONS AMONG MOUSE T LYMPHOCYTES IN THE RESPONSE TO PHYTOHEMAGGLUTININ*

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A synergistic interaction between mouse thymocytes (ThC)[1] and T lymphocytes of the peripheral lymphoid organs has been observed in vitro in the mixed lymphocyte reaction (1,2) and in the generation of cytotoxic T lymphocytes (2–4), and in vivo in the graft vs. host reaction (5). This report describes a type of synergistic response existing in vitro in the proliferative response to phytohemagglutinin (PHA) of a mixture of lymph node cells (LNC) and ThC, which provides a simple model for investigating some forms of T-cell interactions.

Materials and Methods

Mice. CBA/H-T6T6 and CBA/Ca were purchased from The Jackson Laboratory, Bar Harbor, Maine, and bred in our colony.

Cell Preparation. LNC were prepared from a pool of peripheral and mesenteric lymph nodes taken from 3- to 5-mo old mice. Lymph nodes were first teased in Hanks' balanced salt solution (HBSS) and the pieces of tissue sedimenting at 1 *g* were processed further in a ground glass homogenizer (Tissue Grinder, Tenbroeck; Bellco Glass Inc., Vineland, N. J.). The cell suspension was washed twice in HBSS and finally resuspended in culture medium. ThC were prepared by teasing the thymuses of 4- to 8-wk old donors with forceps. Depletion of LNC and ThC in adherent cells was carried out either by plastic adhesion (6) or by the use of nylon wool columns (7). Spleen cells depleted in T lymphocytes were prepared by treatment with mouse anti-θ C3H and C′ as described elsewhere (8). ThC of high density were prepared on discontinuous bovine serum albumin (BSA) gradients (9). Irradiated cells received 800–1,000 rad from a cobalt^{60} source, delivered in 1–2 min.

Cultures. Cultures were performed in tubes (12 × 75 mm; Falcon Plastics, Div. of Bio-Quest, Oxnard, Calif.) containing 1 ml of the culture medium described by Click et al. (10) supplemented with 0.5% mouse serum. Two kinds of PHA were used, with identical results: a crude saline extract from *Phaseolus vulgaris* and a purified PHA (Ref. MR 68; Wellcome Research Laboratories, Beckenham, Kent, England). The optimal stimulating dose was determined empirically, which for the latter was around 1 μg/ml. This optimal dose was not markedly modified for cultures containing different numbers of cells (from 2×10^5 to 3×10^6).

Measurement of [^{3}H]Thymidine (^{3}HTdR) Incorporation. Cultures were pulsed 4 h with 1 μCi of ^{3}HTdR (1 Ci/mmol, Radiochemical Centre, Amersham, England) and harvested with a multiple

* Supported by grant no. 3.797.72 of the Fonds National Suisse pour la Recherche Scientifique.

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; HD, high density; ^{3}HTdR, [^{3}H]thymidine; LD, low density; LNC, lymph node cells; MLC, mixed lymphocyte culture; MTLA, mouse T-lymphocyte-specific surface antigen; PHA, phytohemagglutinin; ThC, thymocytes.

TABLE I

Synergism between LNC and ThC (or B Cells) in the Response to PHA

Cells per culture ($\times 10^{-6}$)		^{3}HTdR incorporation	Synergism*	Caryotypes (% of LNC)
LNC (T6T6)	Other cells (CBA/ca)			
Two representative experiments, arrested on the 3rd day of culture.		*cpm/culture*‡		
1.0	0	74,300	—	—
0	1.0 ThC	1,100	—	—
0.2	0	2,100	—	—
0.2	0.8 ThC	83,000	5.3	72
0.2	0.8 irradiated ThC	97,000	6.5	ND
1.0	0	52,000	—	—
0	1.0 B cell§	1,800	—	—
0.2	1.0 B cell	66,000	5.8	97

Degree of synergism and of ThC proliferation in response to PHA on various days in cultures containing a mixture of LNC (0.2×10^6) and ThC (0.8×10^6).

Culture arrested on *day:*	2	3	4
Synergism*	2.3 (2–3)‖	5.7 (3–9)	4.6 (3–9)
% of mitoses of ThC origin	22 (11–26)	33 (11–50)	35 (16–52)

* Synergism is expressed as the ratio of the ^{3}HTdR incorporated in the LNC-ThC mixture to that response expected from the populations alone ($^1/_5$ the response of 1×10^6 LNC plus $^4/_5$ of the response of 0.8×10^6 ThC).

‡ ^{3}HTdR incorporation in cultures without PHA (background) were in all cases below 1,000 cpm/culture.

§ Spleen cells depleted in T lymphocytes as described in the Materials and Methods.

‖ Mean of at least four experiments: the range of values observed are in parentheses.

cell culture processor (Skatron AS, Liebyen, Norway). The media were allowed to pass through glass fiber filters which retain particulate material. After drying, the filters were placed in vials containing scintillation fluid and counted in a Beckman L-250 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The results presented are the mean counts per minute of duplicate or triplicate cultures.

Count of Blasts per Culture. The counts were done according to Häyry et al. (11) on cytocentrifuged Giemsa-stained slides (Shandon Scientific Co., London, England). Blasts were defined as cells with a nuclear diameter larger than 10 micron.

Caryotypic Analysis. This was performed on cells pooled from duplicate cultures, according to the standard technique of Moorhead and Nowell (12); 40 to 50 mitoses were scored when the results are expressed as a percent.

Immunofluorescence and Autoradiography. Staining of T cells with anti-mouse T-lymphocyte-specific surface antigen (MTLA) antiserum (13) and autoradiography were performed as described previously (14).

Results and Discussion

Synergism in the Response to PHA of Mixture of LNC and ThC. The response to PHA of cultures containing a mixture of 0.2×10^6 LNC and 0.8×10^6 ThC exhibits a strong synergism when compared to the response of 1×10^6 LNC or ThC, since the ^{3}HTdR incorporated in the LNC-ThC mixture is about five times higher than the response expected from the response of LNC and ThC alone (Table I). To explore the origin of proliferating cells, cultures containing

0.2×10^6 LNC and 0.8×10^6 ThC, syngeneic with each other but differing by the T6 chromosome, were stimulated with PHA and the caryotype of the mitoses observed on sequential days was analyzed. The results (Table I) indicated that the ThC, which alone are nearly unresponsive to PHA, had been induced to proliferate in the LNC-ThC mixture. However, ThC proliferation was by far in itself not sufficient to account for the observed synergism. Indeed, since the percent of ThC mitoses was inferior to that of LNC (while the number of ThC in the culture was four times that of LNC) it is obvious that the response of 0.2×10^6 LNC in the LNC-ThC mixture was much higher, on a per cell basis, than the response of 1×10^6 LNC alone. Thus, if the fraction of the ^{3}HTdR incorporated in proliferating LNC (estimated from the percentage of mitoses of LNC origin) is compared to $^1/_5$ of the incorporation of cultures established with 10^6 LNC, it can be calculated that on the 3rd day, the LNC proliferative response may be up to 10 times higher in the LNC-ThC culture. Immunofluorescence analysis with an anti-MTLA antiserum of the blasts present in these various cultures showed that more than 90% of them were of T nature. Thus, the presence of both LNC and ThC in cultures stimulated by PHA leads, under certain conditions, to a double reciprocal "synergism": (*a*) the presence of LNC allows the ThC, which alone are almost unresponsive, to proliferate; (*b*) the presence of ThC increases the proliferative response of LNC to PHA. The following experiments were devised to analyze these two types of interactions.

Enhancing Effect of LNC on Response of ThC in the Presence of PHA. The induction of ThC proliferation poses two questions: the nature of the responding ThC and that of the proliferation inducing agent. ThC of high density, which correspond mainly to cortical ThC, were selected on a BSA gradient and cultures were performed with 0.8×10^6 of these ThC and 0.2×10^6 LNC. While the effect of these small ThC on the proliferative response of LNC to PHA was similar to that of unselected ThC (Fig. 2), caryotypic analysis showed that they did not proliferate (not shown). This however could result from an effect of the BSA on these cells, since incubation of unselected ThC in 30% BSA for 60 min rendered them unable to proliferate in the same culture conditions. A modification of the in vitro behavior of rat lymphocytes, after their incubation in BSA, has also been reported in other instances (15). Cortisone-resistant ThC, which appear to correspond to medullary ThC, could not be tested to explore the nature of the cell involved in the LNC-ThC synergism, since these cells repond by themselves to PHA. Thus, the nature of the proliferating ThC remains undetermined.

The factor(s) promoting the proliferation of ThC could be released by macrophages, especially if activated by a product(s) released by the stimulated lymph node T cells (16). LNC and ThC were depleted in macrophages by two different techniques (see Materials and Methods) and these treated cells, alone or in LNC-ThC combination, showed no significant difference in their response to PHA, amount of synergism, or proliferation of ThC when compared with cultures of untreated cells (not shown). Thus, it seems more likely that it represents a form of T-cell cooperation, probably induced by a soluble mediator released by the lymph node T cells under the influence of PHA (17). This is supported by preliminary observations showing that ThC (1×10^6) proliferate when cultured in the supernate of a 24 h culture of 3×10^6 LNC and PHA, while they do not in the supernate of a similar culture containing ThC in place of LNC

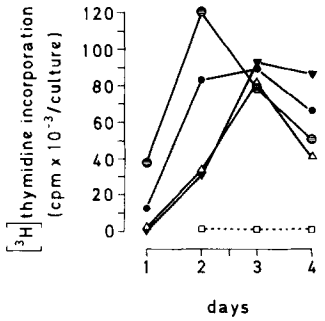

FIG. 1. The response of different concentrations of LNC to PHA on varying days. (⊜), 3 × 10^6 LNC/culture; (●), 1 × 10^6 LNC/culture; (▼), 0.2 × 10^6 LNC and 0.8 × 10^6 irradiated ThC; (△), 0.2 × 10^6 LNC and 0.8 × 10^6 ThC; and (□), 1 × 10^6 ThC alone. Background values (no PHA) are not represented but are below 2,000 cpm.

(12,150 vs. 150 cpm, mean of two similar experiments, 3rd day of culture). Depletion of LNC in adherent cells did not modify this effect.

Enhancing Effect of ThC on LNC Proliferation

NATURE OF THE CELLS REQUIRED FOR OBSERVING THE ENHANCING EFFECT. The observation mentioned above concerning the effect of nonproliferating high density-ThC suggested that ThC proliferation is not a necessary component of the "synergism." This was confirmed using irradiated ThC (Table I and Fig. 1). To explore if this effect of ThC is specific for cells of this origin, B lymphocytes, prepared from a spleen suspension treated with anti-θ and C′, were used in place of ThC (Table I). They were found to have an identical effect on LNC proliferation, while they themselves proliferated little in these conditions [in contrast to what has been reported, in other conditions of culture, on the response to PHA of mixtures of thymus-derived and independent lymphocytes (18)]. This indicated that the enhancing effect observed is specific neither for ThC nor for T cells and suggested the possibility that it may result from the dilution of the LNC.

INFLUENCE OF THE LNC CONCENTRATION ON THE RESPONSE TO PHA OF CULTURES CONTAINING LNC OR LNC-ThC MIXTURES. A low concentration of LNC such as 0.2 × 10^6 LNC/culture, gave variable responses, unless 0.8 × 10^6 ThC were added, and at 0.1 × 10^6 LNC/culture or below, addition of ThC (or B cells) was always necessary to observe a proliferative response (Fig. 2). Thus, a low number of LNC is unable to express its proliferative potentialities, unless other cells are added. If those cells play only a role of "feeder layer," (improving the survival of a small number of cells at the beginning of the culture) or if in addition, they also play a role in the presentation of PHA, ["matrix hypthesis" of PHA stimulation (19), in which the stimulating factor is the contact between cells whose membranes bear PHA, rather than the binding of PHA itself] has not been explored.

Concentrations of LNC between 0.5 and 2 × 10^6/culture, which were fully able to respond alone, gave similar responses to PHA on the 3rd day, while higher concentrations of LNC (3 × 10^6 or more) gave an increase of ^{3}HTdR incorporation culminating on the 2nd day with a rapid decline afterwards (Fig. 1). Fig. 2 shows

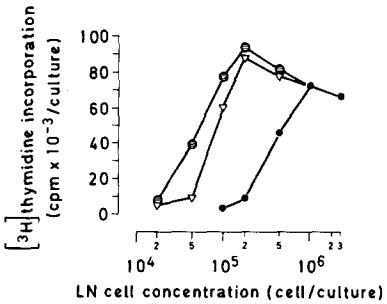

FIG. 2. Response to PHA as a function of the concentration of the LNC, alone or in LCN-ThC mixtures, on the 3rd day of culture. (⊜), mixture of LNC and ThC cells with increasing number of LNC in a total number of 10^6 cells/culture. (▽), mixture of LNC and high density ThC, increasing number of LNC in a total number of 10^6 cells/culture. (●), response of LNC alone, with a variable number of cells per culture.

TABLE II

Autoradiographic Study of the Transformed Cells in Cultures Initiated at Different Cell Concentrations

Culture (+ PHA)	Blasts per culture		Labeled blasts	
	Total	Labeled*	30 min ^{3}HTdR pulse	7 h ^{3}HTdR pulse
			%	%
2×10^6 LNC	1,680,000	520,800	17	31
2×10^5 LNC + 0.8×10^6 irradiated ThC	562,000	488,900	48	87

Representative experiment, 3rd day of culture.
* Labeled blasts per culture, after a 7 h pulse with ^{3}HTdR.

that the LNC response to PHA is no longer proportional to the number of cultured cells above 2×10^5 LNC/culture. Beyond that concentration, the response does not increase further, when expressed per culture, and decreases drastically when related to the number of cultured cells. Thus, the most durable proliferative response to PHA is obtained with a LNC concentration not exceeding 2×10^5 cells/culture, while at higher concentrations, a higher number of cells start to proliferate but their proliferation stops earlier. A similar influence of cell concentration on the response to PHA of human peripheral blood lymphocytes has recently been reported (20).

AUTORADIOGRAPHY OF PHA BLASTS IN CULTURES INITIATED AT DIFFERENT CONCENTRATIONS: EVIDENCE FOR A PHENOMENON OF "BLAST SATURATION DENSITY." Cultures made of 2×10^6 LNC (high density [HD] culture) or 2×10^5 LNC and 0.8×10^6 irradiated ThC (low density [LD] culture) were compared on the 3rd day of culture, using ^{3}HTdR incorporation, number of blasts, and number of blasts labeled after a pulse of ^{3}HTdR (Table II). Although there was a similar level of ^{3}HTdR incorporation in HD and in LD cultures (not shown), the HD culture contained about three times more blasts (about 90% of the cells in these cultures on the 3rd day were blasts) but while almost all the blasts were

labeled in the LD culture after a 7 h pulse, only 30% were labeled in the HD culture. Longer ^{3}HTdR pulses of 12 h or 18 h did not significantly modify these percentages. Thus, both types of cultures contained about the same number of labeled blasts (in good correlation with the ^{3}HTdR incorporation), while a majority of the blasts were not proliferating in the HD culture. Since ^{3}HTdR incorporation indicates that on the 2nd day of culture, the number of proliferating cells is much higher in HD cultures than in LD cultures (Fig. 1), the most likely interpretation of these results is that when a certain concentration of proliferating cells is reached ("saturation density"), an increasing number of them leave the proliferative cell cycle.

Thus, these results, showing the critical role of LNC concentration (and probably of blast density) on the extent and duration of LNC proliferative response to PHA, support the idea that the effect of ThC on LNC proliferation is indirect and results mainly from the dilution of the LNC. That ThC do not directly help LNC proliferation, except when replacing an equivalent number of LNC, was already suggested by the finding that LNC proliferate much more strongly when in LNC-ThC mixtures made with nonproliferating ThC (Fig. 1). Furthermore, when ThC were added to a concentration of LNC able to respond alone, the cellular proliferation was unchanged. For example: ^{3}HTdR incorporation on the 3rd day of a culture containing 1×10^6 LNC was 50,000 cpm, and 47,000 cpm in a parallel culture containing 1×10^6 LNC and 1×10^6 ThC.

In conclusion, the synergism observed in the response to PHA of a mixture of LNC and ThC consists of several components: (*a*) a real synergism exerted on ThC, which leads them to proliferate in a condition where they are unresponsive when alone; (*b*) a synergism exerted on LNC permitting a response to PHA when they are in a concentration too low to proliferate unaided. The mechanisms of this effect ("feeder layer" and "matrix presentation" of PHA) have not been explored; and (*c*) a "synergism" exerted on the duration of the LNC proliferation, which is only apparent and results from the dilution of LNC by cells which are less (or not) able to proliferate in these conditions. It is proposed that this dilution retards the time when the culture reaches a critical concentration of blasts, above which proliferation progressively stops. Combination of factors (*b*) and (*c*), in both of which ThC play only a nonspecific role, leads to the best conditions for prolonged T-cell proliferation under the influence of PHA.

These findings have interesting implications on the possibilities of T-cell interactions, synergistic or suppressive, in vitro and in vivo. Concerning T-cell synergism, it appears that the stimulation of some type of T cells may lead T cells of another variety to proliferate in conditions where they are usually unresponsive; proliferation of ThC in LNC-ThC mixtures happens not only with PHA, but also in mixed lymphocyte culture (MLC) (reference 2, and unpublished observation). The T-cell type triggered in this type of T-cell synergy is probably found not only in the thymus, but also in the peripheral lymphoid organs, notably the spleen (21,22). However, in the MLC, as in the response to PHA, the most important component of the synergism consists of a much stronger response of LNC in the LNC-ThC mixture (reference 2, and unpublished observation). Whether this is due to a specific enhancing effect of ThC (1,

3) or can also be performed by other cells, such as B cells (23), has yet to be resolved.

The evidence provided by the present experiments, that too high a density of T blasts has a suppressive effect on further blast division might have implications for the understanding of a number of in vitro responses. If clonal proliferation leading to differentiation is important for the generation of cells displaying a specific effect, such as, for example, cytotoxic T lymphocytes, simultaneous proliferation of too large a number of cells would depress the specific immune response, since it would decrease clonal proliferation. An example of this situation might be the suppression of the generation of cytotoxic T lymphocytes by the addition of concanavalin A to spleen cultures (24), or, more simply, cultures performed at supraoptimal cell density. In this respect, it should be evident that the optimal condition for cell proliferation in vitro, defined as that which allows the more prolonged clonal expansion of the triggered cells, cannot be determined simply on the basis of the increase in DNA synthesis or in the number of effector cells per culture, as is usually done, but requires also that the response be related to the number of initially cultured cells. Finally, whether a high concentration of T blasts in a lymphoid organ would have the same suppressive effect on their proliferation is not known: if it were so, it might account for a number of the effects of suppressor T cells in vivo (25).

Summary

A synergistic effect in the proliferative response to phytohemagglutinin (PHA) can be observed in cultures containing a mixture of mouse CBA/Ca lymph node cells (LNC) and syngeneic CBA/T_6T_6 thymocytes (ThC) when compared to cultures containing only one cell type. This effect was analyzed, at various days of culture and in LNC-ThC mixtures of different ratios, by comparing the origin of the cells in mitosis (detected by caryotypic analysis), the stimulation of DNA synthesis, the number of blasts, and the percentage of blasts labeled after pulses of [^{3}H]thymidine (detected by autoradiography). The following conclusions were reached: (*a*) ThC are induced to proliferate by the presence of LNC, while they are almost unresponsive to PHA when cultured alone; and (*b*) the strongest "synergistic" effect is exerted on LNC, whose proliferation is markedly enhanced. Evidence is presented that this last effect is not specific to the presence of ThC, but results from a dilution of LNC which retards the time when the culture reaches a critical concentration of blasts, above which proliferation progressively stops. Thus, conditions of culture allowing the response to PHA of a low concentration of LNC leads to the most prolonged T-cell proliferation. These observations may be relevant to the types of T-cell interactions, "synergistic" or "suppressive," occurring during in vitro or in vivo immune responses.

We would like to thank Ms. M.-T. Kaufmann for her excellent technical assistance.

Received for publication 2 September 1975.

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