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The production of a soluble human T-lymphocyte derived factor which substitutes for helper T lymphocytes in the *in vitro* production of immunoglobulin

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Summary. A soluble factor(s), human T-lymphocyte derived help factor (HHF), generated following pokeweed mitogen (PWM) activation of irradiated human peripheral blood T lymphocytes was shown to substitute partially for T-lymphocyte helper activity in the T-lymphocyte dependent PWM-stimulated synthesis of immunoglobulin by B cells. The degree of help provided was proportional to the number of cultured irradiated T-lymphocytes producing factor as well as the amount of factor added to the B-cell culture. The helper effect was equally provided by HHF syngeneic and allogeneic to the B lymphocyte. Although there was little stimulation of total protein synthesis, the synthesis and secretion of IgG, IgM and IgA were all stimulated three- to ten-fold by this factor. The B cells required a minimum of 40–55 h exposure to the factor from the initiation of the culture for an increase in Ig synthesis on day 5 to be observed. Addition of HHF to B cells pre-incubated with PWM for different time intervals showed that a maximum helper effect was exerted when the factor was added on day 0. Addition on day 1 provided less than 20% of maximum help. The factor did not promote significant increases in either B-cell or T-cell cell division.

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INTRODUCTION

Previous studies on the *in vitro* stimulation of immunoglobulin (Ig) synthesis in human lymphocytes by pokeweed mitogen (PWM) have demonstrated an absolute requirement for T lymphocytes which exert a helper effect by promoting both division and differentiation of B lymphocytes (Saxon, Stevens & Ashman, 1977; Keightly, Cooper & Lawton, 1976; Janossy, Gomez de la Concha, Luquetti, Snajdr, Waxdal & Platts-Mills, 1977; Waldmann, Broder, Blaese, Blackman & Strober, 1974.) The T lymphocytes responsible for the helper function are resistant to irradiation and treatment with mitomycin C (Saxon *et al.*, 1977; Keightly *et al.*, 1976).

The collaboration between murine T and B lymphocytes in the *in vitro* production of antibody has been shown to proceed, at least partially, via a variety of soluble molecules released following antigenic or mitogenic stimulation of syngeneic and/or allogeneic T lymphocytes. These soluble T lymphocyte products have been shown either to have specificity for antigen (Taussig, Munro, Campbell, David & Staines, 1975; Isac, Mozes &

Taussig, 1976; Isac & Mozes, 1977) or to act non-specifically in the enhancement of antibody production (Dutton, 1974; Waldmann, Poulton & Desaymard, 1976; Schimpl, Hunig & Wecker, 1974; Askonas, Schimpl & Wecker, 1974). The latter murine factors have been shown to act by primarily enhancing the maturation of B lymphocytes.

In this study we demonstrate that a population of human T lymphocytes, resistant to irradiation and mitomycin C, produce a soluble factor(s) (HHF) following activation with PWM. This HHF enhances the *in vitro* production of Ig by B lymphocytes in the absence of T lymphocytes. HHF is capable of operating across allogeneic differences and appears to promote B lymphocyte maturation rather than division.

MATERIALS AND METHODS

Lymphocyte separation and cultures

Human peripheral blood leucocyte (PBL) suspensions were prepared by Ficoll-Hypaque differential sedimentation of heparinized blood obtained from normal volunteers. T and B lymphocyte fractions were separated by density sedimentation of spontaneous rosettes formed by T lymphocytes and sheep red blood cells (SRBC) pre-treated with 2-aminoethylisothioronium. The B-cell fractions contained $3 \pm 2\%$ E-rosetting cells (eleven experiments). These procedures and the culture conditions have been reported in detail elsewhere (Saxon *et al.*, 1977). The cultures were incubated for 5 days at 37° prior to radiolabelling.

Isolation of radiolabelled immunoglobulin

The procedures for analysis of radioactive Ig have been reported (Saxon *et al.*, 1977). Briefly, following 16 h incubation with [^{35}S]-methionine in culture medium deficient in non-radioactive methionine, the radioactive Ig molecules were isolated from the culture medium by treatment with rabbit antihuman IgM, IgG, IgA, antiserum followed by co-precipitation with *Staphylococcus aureus* bacteria (Kessler, 1975). The washed immune precipitates were reduced, alkylated, and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE).

Following electrophoresis the radioactive Ig molecules were visualized by fluoro-autoradiography (Bonner & Laskey, 1974). A marker sample

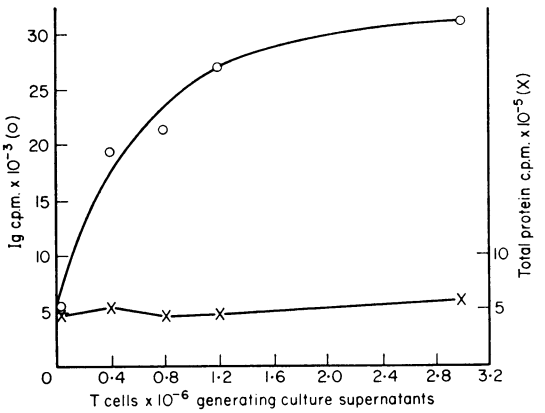

Figure 1. Stimulation of Ig synthesis by the addition of conditioned culture medium derived from increasing numbers of pokeweed mitogen stimulated irradiated T lymphocytes. T lymphocytes obtained by spontaneous SRBC density centrifugation were irradiated (3000 rad) and cultured at different concentrations for 1 day in the presence of PWM. The culture supernatants were removed and 0·7 ml of each were added to $0{\cdot}4 \times 10^6$ B lymphocytes (total culture volume 1·5 ml). After 5 days incubation, the lymphocytes were radiolabelled with [^{35}S]-methionine and the radioactive Ig determined following immune precipitation.

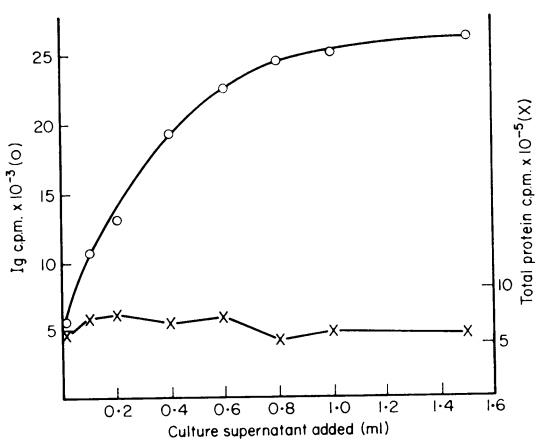

Figure 2. Stimulation of Ig synthesis by addition of increasing amounts of conditioned culture medium obtained from $2{\cdot}0 \times 10^6$ irradiated PWM stimulated T lymphocytes. T lymphocytes obtained by spontaneous SRBC density centrifugation were irradiated (3000 rad) and cultured for 24 h with PWM at a density of 2×10^6 T lymphocytes/ml. The culture supernatant was removed and increasing volumes were added to $0{\cdot}4 \times 10^6$ B lymphocytes (total culture volume 1·5 ml). After 5 days incubation the lymphocytes were radiolabelled with [^{35}S]-methionine and the radioactive Ig determined following immune precipitation.

containing radioiodinated (^{125}I) mu (μ) gamma (γ) and light chains was electrophoresed in parallel with each gel run for molecular weight determinations.

DNA synthesis

Quadruplicate cultures were pulse labelled with 1·0 μCi/ml of [^{3}H]-thymidine (S.A. 2 Ci/mmol, New England Nuclear) for 18 h at various periods during culture. Total radioactivity incorporated into DNA was determined by acid insoluble radioactivity and liquid scintillation counting. The percentage of cells incorporating thymidine was determined by autoradiography (Rodgers, 1967) of cell smears using Kodak NTB2 photographic emulsion.

RESULTS

Production of soluble helper factor by irradiated T lymphocytes

In a previous communication (Saxon *et al.*, 1977) we established the requirement for T lymphocytes in the

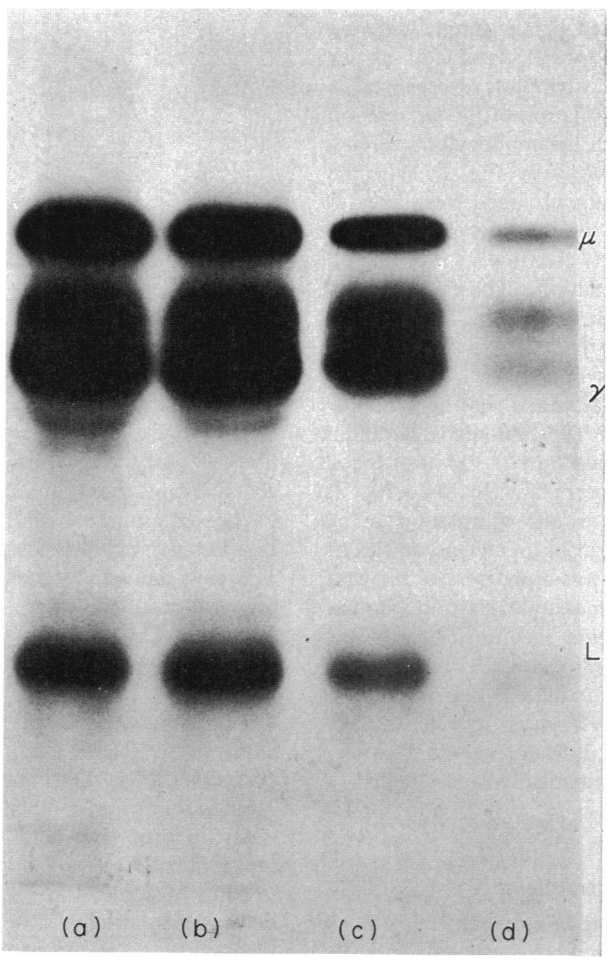

Figure 3. Classes of Ig synthesized by B lymphocytes following treatment with helper factor. The immune precipitates from the samples in Fig. 2 were reduced, alkylated, and separated by SDS–PAGE. The Ig molecules were visualized by fluoro-autoradiography. Exposure time, 4 days. (a) B cells+1·4 ml HHF; (b) B cells+1·0 ml HHF; (c) B cells+0·6 ml HHF; (d) B cells alone: μ, γ, and L indicate the mobility of bands following reduction and alkylation of radio-iodinated marker IgM and IgG proteins.

PWM stimulation of Ig synthesis by B lymphocytes and demonstrated the helper function was not sensitive to irradiation or mitomycin C treatment.

To assess whether all, or part, of the T lymphocyte help was mediated by soluble factors, two titrations of culture supernatant obtained from PWM activated T lymphocytes were performed. First, a constant volume of culture supernatant (0·7 ml) obtained from increasing numbers of irradiated T lymphocytes which had been stimulated with PWM for 1 day was added to $0{\cdot}4 \times 10^6$ B lymphocytes. After 5 days cultivation at 37°, the cultures were radiolabelled with [^{35}S]-methionine for 16 h and the radiolabelled biosynthesized Ig measured following immune precipitation. Culture medium from as few as $0{\cdot}4 \times 10^6$ irradiated T lymphocytes caused a greater than four-fold stimulation of Ig synthesis on day 5 when compared with B lymphocytes receiving culture medium and PWM alone (Fig. 1). With the addition of culture medium obtained from greater numbers of T lymphocytes, there was a further increase in Ig synthesis eventually reaching a maximum six-fold stimulation. There was little change in the synthesis of total protein. The titration of the help factor was extended by examining the production of Ig by B lymphocytes following the addition of increasing amounts of help factor generated by $2{\cdot}0 \times 10^6$ PWM stimulated irradiated T lymphocytes (Fig. 2). Addition of 100 μl of the T lymphocyte cultured supernatant to $0{\cdot}4 \times 10^6$ B lymphocytes caused a two-fold stimulation of Ig synthesis. With the addition of greater quantities of culture supernatant there was a progressive increase in Ig synthesis reaching a maximum six-fold stimulation when the entire culture medium (1·5 ml) was from activated T lymphocytes. Total protein synthesis was minimally stimulated. The stimulation of Ig production by HHF was reflected in an increased synthesis of IgG, IgM and IgA as shown by SDS–PAGE of the biosynthetically radiolabelled Ig molecules (Fig. 3).

Syngeneic *v.* allogeneic helper factor

Our previous studies of the *in vitro* human B–T cell collaboration demonstrated that genetic identity between T and B cells was not required for maximum enhancement of Ig synthesis. Similar experiments were performed using HHF derived from different individuals. The stimulation of B cell Ig synthesis was not significantly different whether HHF was produced by syngeneic or allogeneic T lymphocytes (Table 1).

Table 1. Comparison of syngeneic and allogeneic helper factors. Soluble factors were harvested from PWM stimulated (16 h) irradiated T lymphocytes (2×10^6 ml) from two individuals. Increasing quantities of the two HHF were then incubated 5 days with B lymphocytes from one of the original donors prior to radiolabelling and determination of synthesized total protein and Ig

	T cells ($\times 10^6$)	ml factor	Ig (c.p.m. $\times 10^4$)	Total protein (c.p.m. $\times 10^5$)
	0·4	—	18·1	24·9
	—	—	1·3	9·5
Syngeneic	—	0·05	3·7	13·9
	—	0·2	4·8	16·5
	—	0·4	5·8	18·5
	—	0·8	7·7	17·5
	—	1·0	8·2	15·5
Allogeneic	—	0·05	2·3	11·5
	—	0·2	4·4	16·5
	—	0·4	6·4	17·5
	—	0·8	7·9	18·0
	—	1·0	12·0	23·5

The maximum stimulation of Ig synthesis by HHF, either syngeneic or allogeneic, was consistently less than 60% of the total helper effect capable of being generated by the addition of intact T lymphocytes to the same number of B cells. This indicated that the factor preparation was not capable of totally replacing the activity seen with intact T-lymphocyte preparations.

Kinetics of B-cell activation by HHF

The previous experiments suggested that the primary effect of the HHF was selectively to increase the synthesis of Ig. This maturation may be initiated rapidly following contact with the B cell. Alternatively, limited B-cell differentiation may proceed independently of the helper factor during the initial portion of the culture time and require the helper activity only briefly for terminal differentiation. To distinguish between these alternatives, experiments were performed to determine (1) when the B cells were receptive to HHF, and (2) the minimum time required for contact between B cells and HHF in order to stimulate Ig synthesis.

In the first experiment B cells were initiated

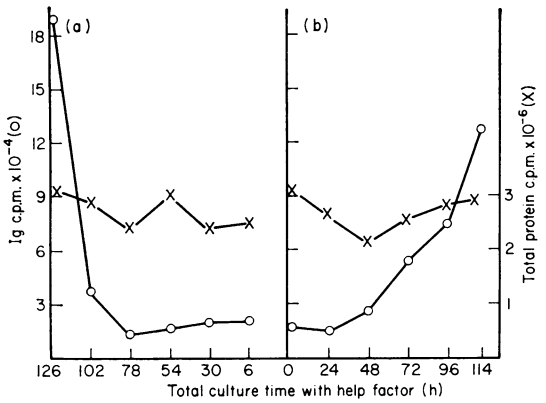

Figure 4. Time course of B lymphocyte stimulation by help factor. (a) Help factor (0·7 ml) generated from one day incubation of PWM stimulated T lymphocytes ($2{\cdot}0\times10^6$/ml) was added to cultures of B lymphocytes ($0{\cdot}4\times10^6$) at 24 h intervals following initiation of the cultures. After 5 days incubation, the cultures were incubated for 16 h with [^{35}S]-methionine and the radioactive Ig and total protein determined. (b) B lymphocytes ($0{\cdot}4\times10^6$) were incubated with 0·7 ml help factor generated by 1 day incubation of PWM stimulated T lymphocytes. At selected intervals (h) the cells were centrifuged, the original conditioned culture medium removed and replaced with culture medium without help factor. Incubation was continued until day five when the cultures were biosynthetically labelled and the radioactive Ig and total protein determined.

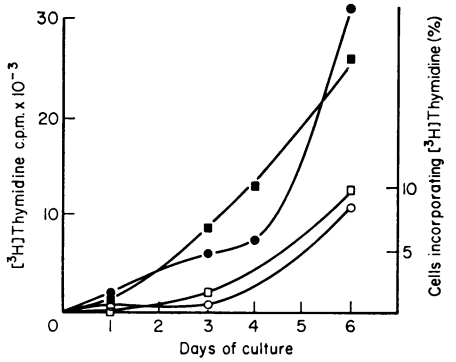

Figure 5. DNA synthesis in B lymphocytes treated with help factor. Parallel cultures of $0{\cdot}4\times10^6$ B lymphocytes incubated with helper factor (■) or without helper factor (●) were pulsed with 1·0 μCi/ml [^{3}H]-thymidine for 18 h at various intervals of culture and the acid soluble radioactivity determined as in Materials and Methods. Similarly the percentage of cells incorporating [^{3}H]-thymidine after various intervals of culture with HHF (□) or with control medium (○) was determined by autoradiography.

without HHF and at day 0 and subsequent 24 h intervals, HHF was added to duplicate cultures. On day 5, all cultures were radiolabelled for 16 h and the synthesis of Ig determined. Maximum stimulation of Ig synthesis was achieved when the factor was added on day 0 (Fig. 4a). Addition of HHF on day 1 or later showed a greater than 80% reduction in the observed soluble helper activity with no effect on total cell protein synthesis.

To determine whether a brief or a prolonged contact between HHF and the B cells was required for maximum stimulation of Ig production on day 5, the reverse experiment was performed. B-cell cultures were initiated with helper factor on day 0, and at daily intervals, the medium containing the helper factor was removed from duplicate cultures and replaced with normal culture medium. On day 5 all cultures were radiolabelled and Ig synthesis determined. Incubation with HHF for up to 40 h provided little stimulation of Ig synthesis over background levels of B cells alone (Fig. 4b). The presence of the HHF for 40–120 h promoted a six to eight-fold increase in the amount of Ig secreted. Again, total protein synthesis was essentially unaffected.

Effect of HHF on lymphocyte DNA synthesis

The enhanced secretion of Ig by B lymphocytes following addition of helper factor suggested that one of its effects was to promote B lymphocyte maturation. To determine, whether HHF also promoted proliferation of B lymphocytes, the synthesis of DNA in cultures receiving HHF or

Table 2. Effect of HHF on T-cell thymidine incorporation. $0{\cdot}4\times10^6$ lymphocytes were cultured with or without HHF (0·5 ml). On days 2 or 5 the cultures were pulsed 18 h with 1 μCi [^{3}H]-thymidine and the acid insoluble radioactivity determined. On day 5, additional cultures were radiolabelled with [^{35}S]-methionine for the determination of total protein and Ig synthesis

	[^{3}H]-Thymidine c.p.m.				
	T cells	T cells +PWM	T cells +HHF	B cells +PWM	B cells +HHF
Day 2	725	275	290	477	593
Day 5	300	2300	2619	4890	6154
% Ig synthesis on day 5	—	0·18	0·3	1·2	4·3

control culture medium was measured. B-lymphocyte cultures containing HHF or fresh culture medium plus PWM were radiolabelled for 18 h with [^{3}H]-thymidine at various intervals. Total DNA synthetic activity was measured by acid insoluble radioactivity while the percentage of cells incorporating tritiated thymidine was determined by autoradiography.

Cultures incubated with HHF initially showed increases in DNA synthetic activity, both in total radioactivity and percentage of cells responding. At termination of the culture period, however, there was no significant difference in the DNA synthetic activity of cultures with HHF and controls (Fig. 5). The observed effect of HHF on B-cell stimulation could result from the stimulation of residual T cells by HHF which subsequently induces B-cell maturation. The effect of HHF on T-cell proliferation was therefore also examined. As shown in Table 2, HHF, under conditions which stimulated Ig synthesis in B cells four-fold, caused no significant increase in T-cell [^{3}H]-thymidine incorporation over PWM control.

DISCUSSION

T-lymphocyte dependent mitogen stimulation of human B cells results in both an increase in proliferation as well as maturation of the Ig producing lymphocytes (Saxon *et al.*, 1977). These two aspects of B-cell differentiation may be independently controlled and modulated by different T lymphocyte subpopulations or active factors derived from these cells. Murine T lymphocyte factors have been described which have antigenic specificity and appear to affect both the maturation and proliferation of B lymphocytes (Taussig *et al.*, 1975; Isac *et al.*, 1976; Isac & Mozes, 1977). Other workers have identified soluble factors obtained from murine T lymphocytes which are highly efficient in promoting B-lymphocyte maturation but do not appear to be directly involved in promoting proliferation (Dutton, 1974; Waldmann *et al.*, 1976; Schimpl *et al.*, 1974; Askonas *et al.*, 1974). These latter murine T lymphocyte derived factors which lead to increased B-cell maturation appear to act on lymphocytes which have received a prior stimulus via contact with antigen.

The HHF described in this paper would appear to be representative of the maturational type of cell to cell signal in that the primary effect is to increase the maturity of B lymphocytes. Addition of HHF to B cells does not promote significant cellular proliferation yet appears to enhance B-cell maturity as determined by the increased Ig synthesis and secretion relative to total protein synthesis. The effect of the factor on only one phase of B-cell activation is further suggested by HHF only partially substituting for intact helper T-cell function even at saturating doses.

The possibility that HHF exerts its effect by inducing proliferation of residual T cells in the B-cell fraction, which in turn stimulate B cells, would appear unlikely as cultures of T cells (>95% E-rosettes) do not respond to HHF with increased [^{3}H]-thymidine incorporation.

The temporal involvement of HHF in the development of the resting B lymphocyte into an Ig-secreting lymphoblast seems to be critical in our culture conditions. Our results suggest an early requirement of HHF for maximal Ig production; the addition of HHF 1 day after initiation of B lymphocytes into culture results in a decrease in Ig secretion which does not appear to be entirely due to shorter culture times with HHF (unpublished results). In the absence of HHF, the culture medium which contains both FCS and PWM as potential mitogenic signals may be envisaged as having a negative effect on B-lymphocyte maturation by making the cells either unresponsive to the subsequent addition of HHF or alternatively more responsive to the possible suppressor influences in the HHF preparation. Similarly, Waldman & Munro (1974) reported that for maximal plaque forming cell response the addition of a non-specific factor, derived from supernatants of antigen-stimulated murine spleen cells, was required on day 0 or day 1 of culture.

Continual exposure of the B cells to HHF for 40 h is required to promote the increased synthesis of Ig. After this time interval, the stimulation is proportional to the length of contact between the factor and the B cells. This continuous requirement of helper factor could result from either a multiplicity of help factors acting sequentially on the B cells, or the gradual recruitment of B cells into a condition of receptiveness for the helper factor. This latter possibility appears unlikely in light of the stringent requirement for exposure of the B cells to the helper factor on the first day of culture. If B cells were continuously being made responsive to HHF

such a decrease in B-cell responsiveness to HHF added after day 0 would seem unlikely. The enhancement of B-cells' Ig synthesis by HHF is similar to that provided by intact T lymphocytes in that the stimulatory effect is provided by allogeneic as well as syngeneically derived factors. Furthermore, like intact T-lymphocyte help, the factors promote the increased synthesis of IgG, IgM and IgA. Whether isotype-specific factors exist cannot be discerned from our data. Finally, based on the criteria of radiation and mitomycin C sensitivity as well as susceptibility of the lymphocytes generating the factor to a human anti-helper T-cell antiserum (unpublished results), it would appear that the cells producing the factor belong to the cell populations responsible for intact T-lymphocyte help. Whether additional factors exist that selectively promote B-cell proliferation, or whether the signal is conveyed exclusively by B cell–T cell contact is unknown.

A soluble factor described by Janossy & Greaves (1975) derived from PWM-stimulated tonsillar T lymphocytes was first shown to enhance Ig production as demonstrated by increased Ig-containing lymphoblasts in B-cell culture. Recently factor(s) isolated from PWM stimulated human peripheral blood lymphocytes were shown to stimulate both proliferation and differentiation of tonsillar B lymphocytes (Insel & Merler, 1977). This would suggest that both of these aspects of B-cell differentiation may be mediated by soluble factors.

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