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Helper activity of T lymphocytes which have been stimulated by keyhole limpet haemocyanin *in vitro*

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Summary. Details are given of a system for keyhole limpet haemocyanin (KLH)-induced DNA synthesis by murine T lymphocytes *in vitro*. Lymph node T cells from mice primed with KLH and *Bordetella pertussis* were stimulated with KLH under the defined conditions, and it was found that such cultured cells exhibited substantial non-specific helper activity. In contrast similarly primed T cells which had not been cultured showed only antigen-specific help. It is concluded that proper account should be taken of non-specific effects when studying the activity of antigen-specific helper cells in *in vitro*.

INTRODUCTION

Primed lymphocytes from several species can be stimulated *in vitro* with soluble antigen to give increased DNA synthesis (Dutton & Eady, 1964; Hersh & Harris, 1968; Seeger & Oppenheim, 1970). However with murine T lymphocytes it proved difficult at first to set up reproducible quantitative systems for antigen-induced DNA synthesis (Osborne & Katz, 1973), although more recently reliable methods have been reported using nylon wool for the T-cell purification (Schwartz, Jackson & Paul, 1975; Corradin, Etlinger & Chiller, 1977). These systems have been useful for investigating the requirements for T-cell activation by soluble protein antigens (Yano, Schwartz & Paul, 1977), but there is little information available concerning the effector functions of the T-cell populations which resulted.

The present paper gives brief details of keyhole limpet haemocyanin (KLH)-induced DNA synthesis by murine T lymphocytes *in vitro* and presents results of investigations to determine whether T cells stimulated in this way exhibited helper activity. T-cell populations, previously primed with KLH *in vivo* and containing antigen-specific helper cells, were cultured with KLH; the results show that under these conditions non-specific helper activity is generated. The significance of this finding is discussed.

Abbreviations: FCS, foetal calf serum; FITC, fluorescein isothiocyanate-conjugated; KLH, keyhole limpet haemocyanin; MEM Hepes, Eagle's minimal essential medium buffered with HEPES; OVA, ovalbumin; TNP, trinitrophenyl group; [^{3}H]-TdR, (methyl-[^{3}H]) thymidine.

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MATERIALS AND METHODS

Antigens

Ovalbumin (OVA, Grade V, Sigma London Chemical Company, Poole, Dorset) and KLH (Calbiochem, Hereford) were used throughout. After conjugation with trinitrophenyl (TNP) using the method of Rittenberg & Amkraut (1966), TNP OVA had 4·5 TNP/mol and TNP KLH had 1000 TNP/mol.

Animals
(BALB/c × B10.D2) F1 mice of both sexes were used; they were bred in the Department of Pathology, Cambridge, from foundation stock (Laboratory Animals Centre, Carlshalton).

Preparation of lymph node T cells
Mice between 6 and 16 weeks old were primed intraperitoneally with 200 μg KLH or OVA together with 10^9 *Bordetella pertussis* organisms (Lister Institute, Elstree, Herts). Four to nine weeks later the mice were killed, mesenteric, inguinal and axillary lymph nodes were removed, teased apart in Eagle's minimal essential medium buffered with Hepes (MEM HEPES), and the cell suspensions were strained through a stainless steel sieve. Subsequently T cells were purified by nylon wool filtration (Julius, Simpson & Herzenberg, 1973). Unprimed T cells were prepared similarly.

Immunofluorescence staining for B and T cells
T cells were tested for purity using fluorescein isothiocyanate-conjugated (FITC) antisera (kindly made available by Dr Bruce Dracott): (a) the FITC anti-mouse immunoglobulin was the $F(ab')_2$ fraction of rabbit anti-MOPC 21 absorbed with mouse red blood cells and liver powder; (b) the FITC anti-T-cell serum was the $F(ab')_2$ fraction of rabbit anti-mouse brain absorbed with mouse red blood cells, liver powder, foetal liver, and nude mouse spleen and lymph node cells. Staining was done in medium containing 5% heat-inactivated foetal calf serum (FCS) and 0·1% sodium azide. Twenty microlitres of cells at 10^7/ml were incubated with 5 μl of FITC antiserum and 75 μl of azide medium for 1 hr at room temperature. The cells were then washed, and scored for membrane fluorescence using a Leitz Orthoplan Ultra-Violet microscope.

Culture medium
MEM (Gibco Bio-Cult, Glasgow) was used with added supplements (Mishell & Dutton, 1967), nucleosides and 5×10^{-5} M 2-mercaptoethanol (Click, Benck & Alter, 1972), and 5% FCS (MEM FCS). The FCS selected for use showed a low background in the absence of added antigen both in the assay for helper activity and in the assay for antigen-induced DNA synthesis.

Assay for antigen-induced DNA synthesis
Lymph node T cells together with antigen as required were cultured in flat bottomed microtitre plates (Linbro) in 0·22 ml MEM FCS/well. The cultures were incubated at 37° in an atmosphere of 10% CO_2, 7% O_2 and 83% N_2. DNA synthesis was assessed by the addition of 20 μl MEM HEPES containing 1 μCi (methyl-[^{3}H]) thymidine ([^{3}H]-TdR), 2 Ci/mmol; Radiochemical Centre, Amersham) to each well 18 hr before harvest. Cultures were collected on GF/A glass fibre discs (Whatman Co., Maidstone, Kent) using a multiple automated sample harvester (Otto Hiller, Madison, Wis.). The discs were mixed with 1 ml scintillation fluid (4 g PPO/1.toluene) and counted in a Nuclear Chicago Isocap Scintillation Counter (background activity 100 c.p.m.).

Statistical evaluation
The data from the DNA synthesis assay were logarithmically transformed (since this usually produced homogeneity of variance) and then tested using analysis of variance. Subsequently each group was contrasted with the others by calculating a value of t from the residual (within groups) variation in order to decide which means were significantly different at the 5% level ($P < 0.05$).

The log–log plots in Fig. 2 were tested by regression analysis using the method of least squares and it should be noted that the data used were not corrected for either the background activity of the liquid scintillation spectrometer or for the spontaneous activity in control cultures.

Assay of helper T-cell activity
TNP-primed B cells were obtained from the spleens of mice which had been injected intraperitoneally with 500 μg TNP OVA in Freund's complete adjuvant and subsequently boosted intraperitoneally with 200 μg of aqueous TNP OVA at 2 monthly intervals. The last boost was given 2 to 6 months later, and 7 to 14 days after the mice were killed. Spleen-cell suspensions were treated with guinea-pig complement and a monoclonal anti-Thy 1·2 serum (Clone F7D5, Olac (1976) Ltd, Bicester, Oxon). Red cells were lysed by the ammonium chloride method (Boyle, 1968) in which the buffer was 0·01 M $KHCO_3$, 0·155 M NH_4Cl and 0·1 mM EDTA.

T cells at a concentration of $2–5 \times 10^7$ T cells/ml were incubated with 25 μg mitomycin C/ml for 20 min at 37° in MEM HEPES (Lefkovits & Waldmann, 1979). Erythrocytes and dead cells were removed by centrifuging over Isopaque and Ficoll (Davidson & Parish, 1975). The cells were then washed extensively to remove residual mitomycin C.

B and T cells were resuspended in MEM FCS together with TNP KLH as required and 10 μl cultures were incubated in Falcon 3034 trays as previously described (Waldmann, Lefkovits & Quintans, 1975; Lefkovits & Waldmann, 1979). IgM and IgG anti-TNP plaque forming cells were determined 5 days later by a modified Jerne assay (Waldmann *et al.*, 1975) using donkey erythrocytes coupled with TNP (Rittenberg & Pratt, 1969); the IgG plaques were developed using rabbit anti-mouse immunoglobulin serum.

RESULTS

Both unprimed and KLH-primed T cells were cultured with a range of concentrations of KLH for 5 days. Figure 1 shows a comparison of the KLH dose–response profiles thus obtained. It is apparent that both cell populations showed a similar increase in incorporation of [^{3}H]-TdR at the highest concentration of antigen. However, at lower doses primed cells gave considerably higher responses than unprimed cells presumably reflecting the stimulation of long-lived memory cells. At 4·5 μg/ml KLH this difference was maximal and it was therefore decided to use this concentration of antigen in all subsequent experiments. It should also be noted that OVA-primed

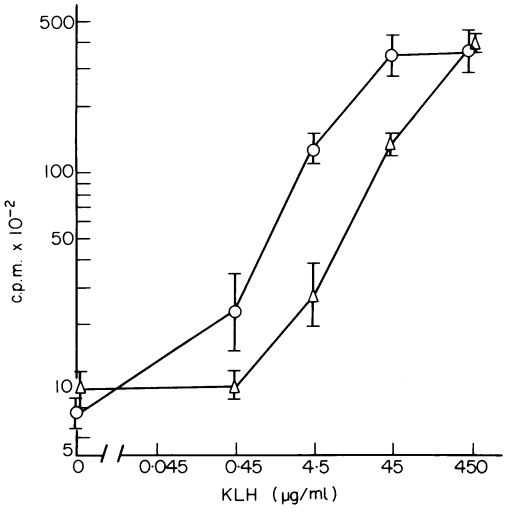

Figure 1. Comparison of the KLH dose–response curves for KLH-primed (o) and unprimed (▵) lymph node T cells. 4×10^5 cells/well were cultured for 5 days and [^{3}H]-TdR was added 18 hr prior to harvest. Results are geometric means of the c.p.m. incorporated by triplicate cultures and bars represent standard deviations.

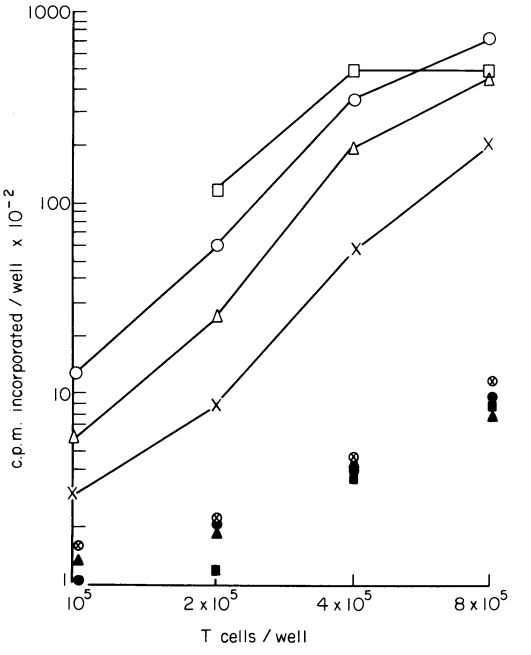

Figure 2. Influence of cell number on the response to KLH made by KLH-primed lymph node T cells. T cells were cultured with 4·5 μg/ml KLH for 3 days (×), 4 days (▵), 5 days (o) and 6 days (□) and with saline for 3 days (⊗), 4 days (▲), 5 days (●) and 6 days (■). Correlation coefficients obtained from linear regression analysis of the results for culture with KLH were: day 3, 0·99 ($P < 0·05$); day 4, 0·99 ($P < 0·05$); day 5, 0·94 ($P > 0·05$ & $< 0·1$); day 6, 0·90 ($P = 0·1$).

lymph node cells gave the same KLH dose–response profile as unprimed cells (data not shown).

The influence of cell number on the response of KLH-primed T cells to KLH was then investigated and Fig. 2 shows some typical results obtained after 3 to 6 days in culture. Linear regression analysis indicated that the log–log plots for the response to KLH after 3 and 4 days in culture were both linear and parallel. The straight lines had a slope of approximately 2, i.e. concentrations in the range 10^5–8×10^5 cells/well showed antigen-induced DNA synthesis which was proportional to the square of the T cell number. In contrast, the data obtained after 5 and 6 days did not fit a straight line and inspection of Fig. 2 shows that at the highest cell concentration the response to antigen reaches a peak on day 5. Finally, the rate of uptake of radioactivity in the absence of added antigen did not change significantly at a given cell density during the experiment.

Figure 3 shows the kinetics of the response to KLH

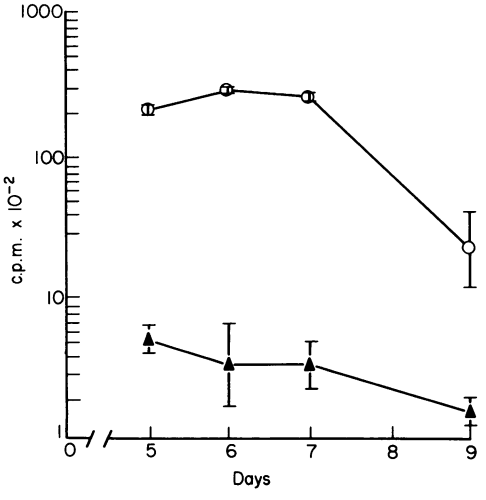

Figure 3. Kinetics of the response to KLH made by KLH-primed lymph node T cells. 4×10^5 cells/well were cultured with 4·5 μg/ml KLH (o) or with saline (▲) and [^{3}H]-TdR was added 18 hr before harvest. Results are geometric means of the c.p.m. incorporated by triplicate cultures and bars represent standard deviations.

made by 4×10^5 KLH-primed T cells/well. It can be seen that the response maintains a plateau between days 5 and 7 and then declines rapidly.

The data presented in Figs 1, 2 and 3 suggested that a reliable system for antigen-induced T lymphocyte DNA synthesis had been established, but the possibility remained that the contaminating B cells in the T cell population (<4%) made a large contribution to the response to 4·5 μg/ml KLH. However, there was no evidence to support this hypothesis since after 9 days culture with KLH: (a) more than 95% of the cells stained with an FITC anti-T cell serum whereas less than 0·6% stained with an FITC anti-immunoglobulin serum; (b) the cells responded by DNA synthesis to concanavalin A but not lipopolysaccharide (data not shown); (c) supernatants from cultured T cells had barely detectable levels of anti-KLH antibody using a sensitive radioimmunoassay (Zollinger, Dalrymple & Artenstein, 1976), whereas comparable supernatants from unfractionated lymph node cells showed a minimum of twenty-five times more IgM and IgG antibodies to KLH.

Having defined the conditions necessary for antigen-induced DNA synthesis by KLH-primed T lymphocytes, cells which had been cultured in this manner for 7 days were tested for helper activity. After

Table 1. The effect of culture with KLH on the helper activity of KLH-primed T cells

	Anti-θ-treated TNP OVA-primed cells ($1{\cdot}5 \times 10^5$/well)	Number of T cells added to each well: *In vivo* primed T cells	Number of T cells added to each well: *In vivo* primed T cells cultured with KLH*	TNP KLH (5 μg/ml)	Total plaque-forming cells	Fraction of non-responding cultures
A	+	—	$4{\cdot}0 \times 10^4$	+	3870	0
	+	—	$1{\cdot}0 \times 10^4$	+	3016	0
	+	—	$3{\cdot}3 \times 10^3$	+	1834	0·17
	+	—	$1{\cdot}1 \times 10^3$	+	938	0·33
	+	—	$4{\cdot}0 \times 10^4$	−	2109	0·07
	+	—	$1{\cdot}0 \times 10^4$	−	1622	0·20
	+	—	$3{\cdot}3 \times 10^3$	−	1295	0·17
	+	—	$1{\cdot}1 \times 10^3$	−	718	0·70
B	+	$1{\cdot}0 \times 10^5$	—	+	4639	0·03
	+	$1{\cdot}1 \times 10^4$	—	+	685	0·43
	+	$1{\cdot}0 \times 10^5$	—	−	232	0·80
	+	$1{\cdot}1 \times 10^4$	—	−	108	0·97
C	+	—	—	+	276	0·67
	+	—	—	−	288	0·73

* $4{\cdot}5 \times 10^5$ T cells per well were cultured with 4·5 μg/ml KLH for 7 days.
† IgM + IgG.

treatment with mitomycin C to inhibit cell division and to abrogate precursor B cell function and certain types of suppressor T-cell function (Lefkovits & Waldmann, 1979), the yield of viable T cells was 10% to 20% of the number initially put into culture. Defined numbers of T cells were then added to a standard saturating number of TNP OVA-primed B cells together with TNP KLH as required. After 5 days in this second culture the number of anti-TNP plaques per well was assayed. The results of a typical experiment are displayed in Table 1, firstly, as the total number of plaque-forming cells observed in 30 microculture wells, and secondly, as the fraction of wells not responding (i.e. those not containing clones of antibody-forming cells).

Part A of Table 1 shows that cultured T cells helped the anti-TNP response in the presence of TNP KLH, and at the highest concentrations of T cells all 30 microculture wells responded indicating that the B cells in the system were not limiting. However, the observed helper activity was largely non-specific since an anti-TNP response was seen in the absence of antigen. There was nevertheless clearly a specific element as well since responses were higher in the presence of TNP KLH.

Part B of Table 1 shows the helper effect of T cells prepared from primed mice that were the siblings of those used 7 days previously to provide the cells tested in Part A. The data show that T cells taken straight from the mouse were less efficient at helping the antibody response than the cultured cells and, moreover, that no helper effect was observed in the absence of TNP KLH. Thus preculturing the primed T cells increased their ability to help non-specifically.

Finally, Part C of Table 1 shows that the number of plaques formed by the B cells alone was low both in the presence and absence of TNP KLH, indicating the lack of functional helper T cells in the B-cell population.

DISCUSSION

The present paper shows that antigen-induced DNA synthesis occurred in cultured lymph node T cells from mice primed with KLH and *B. pertussis*. In contrast, other reported systems have used T cells primed with antigen and Freund's complete adjuvant; Corradin *et al.* (1977) injected mice subcutaneously at the base of the tail and subsequently used the inguinal and peri-aortic lymph nodes, whilst Schwartz *et al.* (1975) immunised mice intraperitoneally and then used thioglycollate-induced peritoneal exudates (PETLES system). However, we found that lymphocytes from mice primed with Freund's complete adjuvant, gave relatively high rates of uptake of [^{3}H]-TdR in the absence of added antigen (data not shown). The present system avoids this problem (Figs 2 and 3) and also has the advantage over the PETLES system that larger numbers of lymphocytes are available for culture since pooled inguinal, axillary and mesenteric nodes are used; consequently considerably less experimental manipulation is needed to obtain suitable numbers of T cells. Lastly, it should be stressed that it is not known whether the same T-cell activity is being measured in each system.

Non-specific helper activity was generated by culturing primed T lymphocytes with KLH (Table 1), but it remains unresolved whether the KLH-specific helper cells present were giving non-specific as well as specific help, or whether these cells can induce non-specific help in other T cells. In either case, preliminary experiments suggested that the process was antigen-dependent; KLH-primed cells cultured without antigen were tested for non-specific helper activity and none was found. Such experiments are, however, technically very difficult and not wholly satisfactory since the yield of cells was very low and variable (1% or less) when KLH was omitted from the cultures.

The exact mechanism of the non-specific helper activity found in the present experiments is not known, but it is possible that factors secreted by the precultured T cells were responsible for the phenomenon. It is well established that factors arising from activated T cells can non-specifically replace the requirement for antigen-specific T cells in the antibody response to T-dependent antigens (Hünig, Schimpl & Wecker, 1977a,b; North, Kemshead & Askonas, 1977; Waldmann & Munro, 1974). Such T cell-replacing factors require antigen to express their activity but the non-specific help reported here occurred in the absence of added antigen. This discrepancy may results from the fact that recently boosted B cells were used in the assay for T-cell help and TNP OVA may therefore be carried over into the cultures as cell bound antigen.

In conclusion, a reliable system for KLH-induced T-lymphocyte DNA synthesis has been established and used to demonstrate that a substantial amount of non-specific help is generated on re-exposing T cells to their priming antigen *in vitro*. This is clearly an

important point for consideration in studies of the nature of the specific T-cell response to antigen.

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