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Induction of human antigen-specific and non-specific helper factors *in vitro*

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SUMMARY

Human peripheral blood leucocytes, cultured for 4–6 days with the appropriate concentration of protein or synthetic polypeptide antigen, yield helper cells.

These cells, after a further 24 hr incubation with the appropriate antigen release helper factors, which are of two types, antigen specific and non-specific; a similar situation to that described in the mouse. These factors are assayed by their effects on the plaque-forming cell response of mouse spleen cells cultures *in vitro*. The potential applications of this new assay for human T cell function are discussed.

INTRODUCTION

With the increasing awareness of the involvement of the immune system in a variety of diseases (reviewed by McMichael & McDevitt, 1977; Rosen, 1975), there has been widespread interest in assessing lymphocyte function in humans with a precision analogous to studies with experimental animals. In mice it has been demonstrated that various functions, such as help, suppression or cytotoxicity are mediated by distinct cells (Cantor & Boyse, 1977; Feldmann *et al.*, 1977a) and thus it is no longer adequate (if it ever was) to assess T cell function by enumeration of circulating T cells or their response to mitogens, which stimulate more than one class of T cell (Dutton, 1972; Rich & Pierce, 1973). In view of the ethical constraints of *in vivo* studies of human T cell function and the impracticality of multiple assays *in vivo*, attempts have been made to induce various T cell functions *in vitro*. Some success has been reported for killer cells, (Newman, Stoner & Bloom, 1977; Carnaud *et al.*, 1977). Because of the paramount importance of antigen specific helper cells in the induction of antibody responses, attempts were made to stimulate human peripheral blood *in vitro* with protein antigens in order to yield helper cells. Since helper cells have been shown to co-operate with B cells via the intermediary action of specific (Feldmann & Basten, 1972) and non-specific factors (Schimpl & Wecker, 1973) which are not strain (Munro & Taussig, 1975) or species specific (Luzzati *et al.*, 1976; Farrar, 1975; Taussig & Finch, 1977), and as human B cell assays are as yet suboptimal, human helper function was assayed *in vitro* on mouse spleen cells. This report describes the induction of a primary helper T cell response, and the release by these cells of antigen specific and non-specific helper factors.

MATERIALS AND METHODS

Human peripheral blood (PBL). PBL from normal healthy volunteers was collected into heparinized syringes, and leucocytes were separated on Ficoll–Triosil gradients (Böyum, 1968) and washed three times in Earle's saline. Viability was assessed with trypan blue. Ten volunteers, aged 28–54 years, participated voluntarily and with informed consent.

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Antigens. The synthetic polypeptide poly (L-tyr, L-glu) - poly(D, L-ala) — poly(L-lysine), abbreviated to (T,G)-A—L, was kindly provided by Prof. E. Mozes, Department of Chemical Immunology, Weizmann Institute, Rehovot, Israel. Keyhole limpet haemocyanin (KLH) was provided by Prof. M.B. Rittenberg, University of Oregon, Portland. Ovalbumin (OA) was obtained from Worthington. Guinea-pig albumin (GPA) was prepared as described earlier (Dwyer & Kantor, 1975). DNP acrylamide beads were prepared as described previously (Feldmann *et al.*, 1974) by Dr D. Parker.

Animals. CBA or C57Bl mice, bred at the ICRF breeding unit, were used at 10–16 weeks of age.

Cultures. The culture medium used was Eagle's minimal essential medium enriched with supplemental amino acids (GIBCO F-15). This was supplemented with either 10–15% donor serum, AB serum, or, more recently, 5% of appropriate batches of foetal calf serum (FCS). Batches suitable for the culture of mouse spleen cells to yield helper cells (Kontiainen & Feldmann, 1973) were also suitable for the culture of PBL. $5–10 \times 10^6$ PBL in 1·0 ml of medium, usually containing donor serum and antigen at 1–5 μg/ml were cultured for 4–6 days in the inner compartment of a Marbrook flask (Marbrook, 1967, Diener & Armstrong, 1967) as described by Kontiainen & Feldmann (1973). No attempt has yet been made to induce helper cells in tubes or dishes. The outer medium usually contained 5–10% calf serum, and sometimes 2-mercaptoethanol (5×10^{-5} M). After the period of culture, in 10% CO_2 in air at 37°C, the cells from a group of cultures were harvested, washed and counted. Cell recoveries averaged 70%. These cells were termed 'helper cells' (HC).

Two assays for the activity of helper cells were used. Initially, helper factor was generated *in situ* in the inner chamber of double chamber Marbrook flasks, the human helper cells separated from mouse spleen cells by a nucleopore membrane of 0·2 μ pore size (Feldmann & Basten, 1972b). The outer chamber contained mouse spleen cells ($1·5 \times 10^7$/ml) in 3·0 ml of HEPES buffered medium, with 0·1–1·0 μg/ml of haptenated antigen. The antigens used in these experiments were trinitrophenylated KLH (TNP KLH), prepared as described by Rittenberg & Amkraut (1969) with 8 groups/10^5 Daltons, TNP ovalbumin (TNPOVA) with 12 groups/mol and TNP guinea-pig albumin (TNP GPA) with 21 groups/mol were prepared as described by Dwyer & Kantor (1975), while DNP (T,G)-A—L had 4 groups/150,000 Daltons. The reservoir of the Marbrook flasks contained bicarbonate buffered Eagle's medium supplemented with 5% FCS and 5×10^{-5} M 2-mercaptoethanol. In later experiments, helper factor was generated in cultures of *in vitro* primed human cells and subsequently assayed in cultures of mouse cells.

Helper factors. Helper factors (HF) were prepared by 24 hr incubation of helper cells ($5–10 \times 10^6$/ml) with 0·1–1·0 μg/ml antigen in Marbrook flasks. Media in both chambers contained 5% FCS. Supernatants were collected after centrifugation of the cells, were filtered through a 0·44 μ Millipore filter, and aliquoted prior to freezing. The helper factors were assayed by their effects when added at various percentages to mouse spleen cells cultured with antigen, measured at day 4, as with mouse HF (Howie & Feldmann, 1977). Factors collected after 24 hr of secondary culture were more potent than supernatants of primary cell culture taken after 4 or 5 days, presumably because of degradation over the prolonged culture period.

Plaque-forming cell (PFC) assays. Since unprimed mouse spleen cells were used for detecting the function of human HC, only IgM PFC were detected. DNP-specific plaque-forming cells were measured by subtracting the PFC obtained in Cunningham type assays with sheep red cells (SRC) either uncoated, or coated with DNP Fab fragments (Cunningham & Szenberg 1968; Strausbach, Silica & Givol, 1970).

Numbers of plaques are expressed as arithmetic means ± standard error of the mean of three cultures. As a control, the response of spleen cells to the thymus independent antigen, DNP acrylamide beads, was measured.

Immunoabsorbents and their use. Immunoabsorbents were prepared using cyanogen-bromide-activated sepharose beads and 1·0 mg of antigen per ml of beads (Porath, Axen & Ernback, 1967). Helper factors at the appropriate dilution were incubated for 1 hr with an equal volume of beads on a rotating platform. The beads were decanted and the supernatant termed 'absorbed HF' (Howie & Feldmann, 1977).

RESULTS

Cooperation of human helper cells with mouse spleen cells across a membrane

Because of the problems of mixing cells of different genetic origin and the consequent potential artefacts, either inhibitory or stimulatory (e.g. Katz, Hamaoka & Benacerraf, 1973), human helper cells (HC) were initially assayed with CBA mouse spleen cells using double chamber cultures (Feldmann & Basten, 1972a). The results of one such experiment are shown in Table 1, using KLH as the inducing antigen. Human PBL cultured with KLH induced an anti-DNP response from mouse spleen cells with TNP KLH, while PBL cultured without antigen in the primary culture had little effect. As a control, mouse HC_{KLH} was used.

Cooperation of human helper cells by the production of helper factors

Mouse helper cells release supernatants with helper activity, termed 'helper factors' which stimulate B cell function (Feldmann & Basten, 1972a; Schimpl & Wecker, 1972). Helper factors were collected from primary cultures of PBL by harvesting the supernatants at the end of the culture period. These were added at 5 or 20% to mouse spleen cells. These supernatants increased the response to the homologous

TABLE 1. Cooperation of human helper cells across an impermeable cell membrane

Stimulus		Response
Helper cells	Antigen	IgM anti-DNP (AFC/culture ± s.e.)
Mouse HC_{KLH} 10^6	TNP KLH	440 ± 170
Mouse HC_{KLH} 3×10^5	TNP KLH	190 ± 200
Human HC_{KLH} 10^6	TNP KLH	720 ± 240
Human HC_{KLH} 3×10^5	TNP KLH	190 ± 100
Human HC_{NIL} 10^6	TNP KLH	20 ± 30
Human HC_{NIL} 3×10^5	TNP KLH	40 ± 24
Human HC_{NIL} 3×10^5	TNP KLH	20 ± 26
Human HC_{NIL} 3×10^5	TNP KLH	42 ± 34
Human HC_{NIL} 3×10^5	DNP beads	930 ± 461

Human PBL were cultured with 1·0 μg/ml KLH for 4 days, CBA mouse spleen with 0·1 μg/ml KLH for 4 days before the cells were washed, counted and placed in double chamber flasks at the numbers stated in the upper chamber. In the lower chamber, 45×10^6 unprimed spleen cells in 3·0 ml were cultured with 0·1 μg TNP KLH or 0·3% DNP beads. Responses were measured at day 4. More than ten experiments of this type have yielded analogous results.

TABLE 2. Specificity of human helper factor

Stimulus			Response
Helper factor	Percentage	Antigen	IgM anti-DNP (AFC/culture ± s.e.)
E_1 Human HF_{KLH}	20	TNP KLH	330 ± 84*
E_1 Human HF_{KLH}	20	DNP(T,G)-A–L	110 ± 40*
Human HF_{TGAL}	20	TNP KLH	115 ± 43*
Human HF_{TGAL}	20	DNP(T,G)-A–L	230 ± 63*
—		DNP(T,G)-A–L	17 ± 6
—		TNP KLH	0
—		Nil	8 ± 6
—		DNP beads	1060 ± 210
E_2 Human HF_{OVA}	5	TNP OVA	359 ± 88*
E_2 Human HF_{OVA}	5	TNP GPA	38 ± 24†
—		TNP GPA	18 ± 14
—		TNP OVA	14 ± 10
—		Nil	9 ± 7
—		DNP beads	730 ± 220
E_3 Human HF_{KLH}	1	TNP KLH	420 ± 23*
E_3 Human HF_{KLH}	1	DNP (T,G)-A–L	40 ± 6†
Human HF_{TGAL}	1	TNP KLH	43 ± 9†
Human HF_{TGAL}	1	DNP(T,G)-A–L	293 ± 20*
—	—	DNP(T,G)-A–L	30 ± 12
—		TNP KLH	40 ± 12
		Nil	30 ± 6
		DNP beads	1467 ± 102

Human PBL were cultured with antigen, (1·0 μg KLH or (T,G)-A–L, 10 μg OVA) and the supernatants collected after 4–6 days (E1 and E2) and used at 20 or 5% as shown. The response of unprimed CBA spleen cells to 1·0 μg/ml TNP OVA, TNP GPA or 0·1 μg/ml TNP KLH was assessed after 4 days. E1 and E2 were cultured with 10^4 responding cells in 1·0 ml in usual Marbrook flasks, while E3 was performed with 3×10^6 responding cells in 'Mini Marbrooks' with a volume of 200 μl. As noted, these respond in an analogous manner per culture, and significantly better per cultured cell input. E3 was performed using T cell-depleted (anti-Thy 1 and complement-treated) spleen cells.

* Statistically significant from background ($P < 0.05$).

† Not statistically significant from background ($P > 0.05$).

TABLE 3. Non-specific helper factors

Stimulus			Response	
			IgM AFC/culture ± s.e.	
Helper factor	Percentage	Antigen	SRC	DNP
—	—	—	96 ± 18	203 ± 48
—	—	SRC	1027 ± 93	—
Human HF_{KLH}	20	SRC	1890 ± 191	—
Human HF_{KLH}	5	SRC	1633 ± 365	—
Human HF_{KLH}	1	SRC	1030 ± 56	—
—	—	DNP beads	87 ± 17	1733 ± 194
Human HF_{KLH}	20	DNP beads	153 ± 81	3010 ± 251
Human HF_{KLH}	5	DNP beads	277 ± 40	2777 ± 503
Human HF_{KLH}	1	DNP beads	73 ± 32	1623 ± 243

Effect of non-specific helper factors on the response to sheep red cells and DNP beads. Human HF_{KLH} was used at 20, 5 and 1% and stimulated SRC and DNP responses at the higher concentrations. Responses of C57Bl/10 mice at day 4 to 3×10^6 SRC/culture or 0·3% DNP beads in 'Mini Marbrooks'.

TABLE 4. Immunoabsorbent analysis of human helper factor

Stimulus			Response	
			IgM response (AFC/culture ± s.e.)	
Helper factor	Percentage	Antigen	DNP	SRC
—		TNP KLH	90 ± 26	147 ± 49
Mouse HF_{KLH}	0·1	TNP KLH	483 ± 101	120 ± 23
Human HF_{KLH}	1	TNP KLH	533 ± 127	77 ± 22
Human HF_{KLH} abs KLH	1	TNP KLH	56 ± 32	33 ± 9
Human HF_{KLH} abs TGAL	1	TNP KLH	547 ± 104	63 ± 26
Human HF_{KLH}	10	TNP KLH	620 ± 55	287 ± 47
Human HF_{KLH} abs KLH	10	TNP KLH	93 ± 56	377 ± 91
Human HF_{KLH} abs TGAL	10	TNP KLH	680 ± 164	360 ± 35
Human HF_{KLH}	10	SRC	—	3383 ± 318
Human HF_{KLH} abs KLH	10	SRC	—	3093 ± 474
Human HF_{KLH} abs TGAL	10	SRC	—	3750 ± 1590
—		SRC	—	1850 ± 168
—		DNP beads	1397 ± 229	—

Immunoabsorbent analysis of helper factors. Two factors can be separated by their differential capacity to bind to antigen columns. Specific factor binds to the appropriate antigen, whilst non-specific factor does not. At high concentrations HF contains detectable non-specific factor, which augments the SRC response. Response of 3×10^6 B10 mouse cells in 200 μl in 'Mini Marbrooks' to antigen and 3×10^6 SRC or 0·3% DNP beads or 0·1 μg/ml TNP KLH or helper factor as shown.

antigen more than to the heterologous antigen (Table 2), but enhancement of other antigens was significant. This is shown more clearly in Table 3.

Helper factors collected as supernatants of the cultured PBL were not always effective if used at 1%. However, supernatants collected after 24 hr re-culture with antigen of washed HC, as described by Howie & Feldmann (1977) with mouse HC, were more potent. These were stimulatory at 1% (Table 2), or sometimes at even lower concentrations. This type of supernatant is now in current use and, when used at 1%, does not contain significant activity of non-specific helper factor (Table 4).

Helper factors are defined by their augmentation of T cells-dependent responses in the absence of T cells. Thus, the effect of HF on responses of T-depleted (anti-Thy1 and complement-treated) spleen cells was ascertained. The stimulatory effect was equivalent in the absence of T cells, demonstrating that these are 'helper' and not 'amplifier' factors, which act on other T cells (Feldmann *et al.*, 1975; 1977b; Tada, Taniguchi & David, 1977), as shown in Table 3.

Verification of the antigen specificity of helper factor by the use of immunoabsorbents

Immunoabsorbents have been highly useful in the characterization of helper factors (Taussig *et al.*, 1975; Howie & Feldmann, 1977). Hence, these were used to verify that the helper factors, which were functionally antigen specific, were in fact specifically bound to an antigen column. Table 4 illustrates typical results using HF_{KLH}, which is bound to KLH but not to (T,G)-A–L immunoadsorbent. It was of interest that absorption of HF at high concentrations (10%) did not remove the non-specific augmentation of the SRC response. This confirms the presence of two distinct helper components in the supernatants of human helper cells.

DISCUSSION

The results presented here indicate that human PBL may respond to antigens, either proteins or synthetic polypeptides, to yield helper cells which augment the response of mouse spleen cell cultures. The use of xenogeneic combinations (human/mouse) is not unique to the current study, but has been reported for non-specific human factors activating mouse B cells (Farrar, 1975), or in the mirror image experiment with mouse HF acting on human B cells (Luzzati *et al.*, 1976; Taussig & Finch, 1977). Attempts are currently in progress to assay human factors on human B cells to further establish their relevance to mechanisms of cell interaction. It is not clear whether the responses described here are primary responses, as these complex protein antigens may be cross-reactive with environmental antigens, or as in the case of ovalbumin, are present in food. Analogous responses with synthetic polypeptides, e.g. (T,G)-A–L, does not prove that these are primary responses, since there may still be cross-reactions. Experiments underway with neonatal cord blood may resolve this question.

The data illustrated here indicate that it is feasible to study helper function from human PBL. The finding of both specific and non-specific helper factors is fully analogous with previous studies in the mouse (Feldmann & Basten, 1972a; Schimpl & Wecker, 1972). This suggests but does not prove that the source of the human HF is from T cells. Cell purification experiments are currently in progress, and the preliminary results suggest that factors do come from T cells.

Results are presented which illustrate the presence of both specific and non-specific helper factors from human PBL acting on mouse spleen cells. The two factors are readily distinguishable, by testing on heterologous antigens and also by absorption in antigen-coated immunoabsorbents (Table 4). The appropriate antigen removed the specific helper activity, but not the non-specific. Apart from the presence of an antigen binding site, nothing is yet known about the nature of the specific helper factor. Immunoabsorbent analysis, so useful in the characterization of mouse helper factors should reveal whether these are products of the HLA region.

While helper factors have been demonstrated in *in vitro* assays in the mouse (Feldmann & Basten, 1972a,b; Schimpl & Wecker, 1972) and rat (Taniguchi & Tada, 1974), there are recent analyses of the genetic restriction of T–B cooperation in the apparent absence of suppressor cells (Sprent, 1978) that suggests that T–B contact may be *in vivo*. It is not possible *in vivo* to conclusively determine whether

factors are operative or cell contact is required. Thus it is not clear whether assays for helper factors fully measure all aspects of T cell help. However, the detection of human helper supports the notion that such moieties are of major significance in T–B cooperation.

The production of specific helper factors in the mouse is under genetic control. Thus, the production of $HF_{(T,G)-A--L}$ *in vivo* or *in vitro* is under H-2-linked control (Munro & Taussig, 1975; Isac & Mozes, 1977; Howie & Feldmann, 1977). In humans there is as yet no clear-cut evidence for the existence & HLA-linked immune response genes (reviewed by De Vries & Munro, van Rood, 1977; McMichael of McDevitt, 1977), but the existence of numerous HLA-linked diseases (reviewed by Svejgaard *et al.*, 1975) led to the concept that HLA-linked immune response genes must exist in humans. The techniques described above, involving a small number of PBL from normal volunteers, who do not need to be injected, should permit a search for human immune response genes. Such an analysis is in progress. A more precise analysis of immune dysfunction in the presence of normal T cell numbers in various diseases is now possible with this technique, and the characterization of human T cell factors and their mechanism of action should facilitate attempts at manipulation of the immune response in immunologically impaired individuals.

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