T CELL GROWTH AND DIFFERENTIATION INDUCED BY INTERLEUKIN-HP1/IL-6, THE MURINE HYBRIDOMA/ PLASMACYTOMA GROWTH FACTOR

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Recently, we purified a new mouse T cell–derived lymphokine that we provisionally designated interleukin-HP1 (HP1)[1] because of its growth factor activity for B cell hybridomas and plasmacytomas (1, 2). This factor, which was identified as a single chain 25–30-kD polypeptide, was subsequently found to be structurally and serologically indistinguishable from the plasmacytoma growth factors produced by macrophages and fibroblasts (3–5). Molecular cloning and sequencing of HP1 cDNA (6) disclosed a very significant degree of homology with the human hybridoma growth factor, now termed interleukin 6 (7–8), that we had previously found to be identical (9) with B cell stimulatory factor 2 (10), 26-kD protein (11) and IFN-β2 (12), and that has now also been identified with the monocyte-derived hepatocyte-stimulating factor (13).

To evaluate the role of HP1/IL-6 in the immune system, we have started to investigate how this molecule affected the response of normal mouse lymphocytes to various stimuli. Recently, we showed that (14) in the presence of IL-1, HP1/IL-6 represented a major stimulus for B cell growth and differentiation. A salient finding of these studies was the observation that the combination of IL-1 and HP1/IL-6 induced B cell responses comparable in many respects to those obtained with established B cell growth and differentiation factors like IL-4 and IL-5.

In the present report we show that HP1/IL-6 also stimulates T cell growth and differentiation, and we provide evidence suggesting that production of this molecule is one of the mechanisms through which accessory cells support polyclonal T cell activation.

Materials and Methods

Mice. All mice used in this study were bred at our local animal facility under specific pathogen–free conditions.

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[1] *Abbreviations used in this paper:* HP1, interleukin-HP1; L-HGF, L929-derived hybridoma growth factor.

T Cell Proliferation Assays. Spleen cells and mesenteric lymph node cells from 10-wk-old female BALB/c mice were cultured in flat-bottomed microtiter wells at a cell density of 3–5 × 10^4 cells/well in DME supplemented with 10% FCS, L-arginine (0.55 mM), L-asparagine (0.24 mM), L-glutamine (1.5 mM), and 2-ME (50 μM). To these cultures we added a costimulator, usually PHA, and the cytokine to be tested. Thymidine incorporation was measured in triplicate cultures on day 4 after a 6-h pulse with 0.5 μCi *methyl*-[^{3}H] thymidine. Optimal responses to HP1/IL-6 required the use of a selected batch of PHA. The best results were obtained with PHA from Difco Laboratories Inc. (Detroit, MI) added at a final dilution of 0.5–1 × 10^{-3}. The response of thymocytes was studied under similar conditions except that we used 6-wk-old mice, a 2 × 10^{-3} dilution of PHA, and higher cell numbers (1.5 × 10^6 for IL-1 and 0.5 × 10^6 for IL-2 and HP1/IL-6). Moreover, the incubation period was reduced to 3 d.

Accessory Cell Depletion. Accessory cell depletion was obtained by passage through nylon-wool columns as described (15) and treatment with anti-I-A^d antibody MKD-6 (16) and rabbit complement (Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada).

Induction of Cytolytic T Cell Responses. C57BL/6 (H-2^b) spleen cells were incubated, in 2 ml of DME supplemented as described above, with 2 × 10^6 irradiated DBA/2 (H-2^d) high-density spleen cells selected by Percoll gradient centrifugation ($1.070 < \rho < 1.088$). This procedure was adopted to reduce the number of macrophages and thus the levels of endogenous HP1/IL-6 in the cultures. Cytolytic activity was measured in a conventional chromium-release assay using P815 mastocytoma (H-2^d) and EL 4 thymoma (H-2^b) as target cells.

Helper T Cell Lines. Helper T cell lines were established from mice immunized with keyhole limpet hemocyanin or human transferrin as described (1). The cell lines were routinely grown with irradiated spleen cells and antigen but without addition of exogenous growth factors. Before use in proliferation assays, the T cells were separated from dead cells by centrifugation over a layer of Lymphoprep (Nycomed, Oslo, Norway).

Cytokines. Human rIL-1α (17) and IL-2 (18) were gifts of Drs. P. Lomedico (Roche Research Center, Nutley, NJ) and W. Fiers (State University of Ghent, Belgium), respectively. HP1/IL-6 and L929-derived hybridoma growth factor (L-HGF) were purified to homogeneity as described earlier (1, 4). IL-4 was partially purified from the supernatant of mouse helper T cell clone TUC2.15 by adsorption on silicic acid, gel filtration, anion exchange, and reversed-phase HPLC. For each cytokine, 1 U of activity was defined as the amount of material required to produce half-maximal proliferation of the appropriate target cell, i.e., hybridoma 7TD1 for HP1/IL-6 (1), cytolytic T cell line CTLL for IL-2 (19), and anti-IgM–stimulated B cells for IL-4. These assays were carried out as previously described (1, 2).

Monoclonal Antibodies. Anti-IL-4 mAb 11B11 (20) was a gift of Dr. W. E. Paul (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). Anti-HP1/IL-6 mAb 6B4 was produced in our laboratory (14). Anti–T cell mAbs used included: (*a*) anti-Thy-1.2 antibody 3A8 also produced in our laboratory, (*b*) anti-T3ε antibody 145-2C11 (a gift of Dr. O. Leo, Laboratory of Animal Physiology, University of Brussels, Belgium) (21), and (*c*) anti-L3T4 antibody GK1.5 (22) and anti-Lyt-2 antibody 53.6.72 (23). All rat mAbs were purified by affinity chromatography using mouse anti–rat κ antibody MARK1 (24), a gift of Dr. H. Bazin (Unit of Experimental Immunology, Catholic University of Louvain, Brussels, Belgium).

Flow Cytometry. Spleen cells and thymocytes were labeled with fluoresceinated GK1.5 and biotinylated 53.6.72 for 45 min at 4° C in 137 mM NaCl, 5 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM $MgCl_2$, 5 mM glucose, 4 mM $NaHCO_3$, 1 mM EDTA, containing penicillin (500 U/ml), streptomycin (1 μg/ml), and 5% FCS, and buffered with 1 mM phosphate (pH 7.4). After centrifugation, the cells were resuspended in the same medium containing phycoerythrin-conjugated streptavidin (Becton Dickinson & Co., Mountain View, CA), and incubated for another 45 min before sorting on an ATC3000 flow cytometer (ODAM, Wissembourg, France). Aggregated and dead cells were excluded on the basis of forward angle light scatter values.

Results

General Remark. HP1/IL-6 was originally quantified on the basis of its growth factor activity for B cell hybridoma 7TD1, which requires ~1 pg/ml for half-maximal growth. It later turned out that the sensitivity of 7TD1 was much higher than that of other HP1/IL-6-dependent cell lines. Plasmacytomas, for example, require ~100 times more HP1/IL-6 than the hybridoma cell line (2). It should therefore be kept in mind that, as a result of this unusual sensitivity, hybridoma growth factor units of HP1/IL-6 may be misleadingly high.

Proliferation of Peripheral T Cells in Response to HP1/IL-6. HP1/IL-6 purified to homogeneity from the supernatant of helper T cell clone TUC2.15 was found to induce a 100-fold increase in the proliferation of spleen cells exposed to submitogenic doses of PHA (Table I, Exp. 1). This response, which was quantitatively similar to that induced by IL-2 and IL-4, was saturable and required ~100 hybridoma growth factor units per milliliter for half-maximal stimulation. The proliferation induced by HP1/IL-6 was completely abolished after treatment with anti-Thy-1 antibody and complement, demonstrating the T cell nature of the responding cells (Table I, Exp. 1). Cell sorting experiments indicated that both the $L3T4^+$ and the $Lyt\text{-}2^+$ subsets participated in this response (Fig. 1).

TUC2.15 supernatant contains no detectable IL-2 but considerable amounts of IL-4. Therefore, we were concerned that in spite of its apparent homogeneity in silver-stained SDS polyacrylamide gels, our HP1/IL-6 preparation might still contain traces of this lymphokine that could be responsible for the observed T

TABLE I

Proliferation of PHA-activated Spleen Cells Induced by HP1/IL-6, IL-2, and IL-4

Exp.	Cytokines	Dose	Responding spleen cells*		
			Unactivated	PHA-activated	PHA-activated T cell–depleted‡
		U/ml			
1	None		160	865	124
	HP1/IL-6	80	224	19,476	103
		400	320	80,528	151
		2,000	297	86,751	200
	IL-2	0.8	313	11,193	356
		4	771	58,303	1,000
		20	1,270	72,224	1,208
		100	1,862	77,022	1,189
	IL-4	4	435	3,771	471
		20	662	26,528	822
		100	654	55,998	760
2	None		259	553	—
	L-HGF	2,000	777	52,785	—

* 5×10^4 spleen cells were stimulated with PHA and the indicated cytokines as described in Materials and Methods. Thymidine incorporation was measured on day 4 (mean of triplicate measurements in cpm, SE <10%).

‡ Cells treated with anti-Thy-1.2 mAb 3A8 and complement.

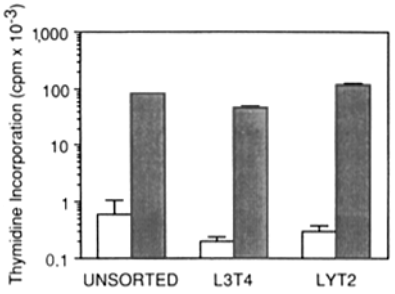

FIGURE 1. T cell subsets responsive to HP1/IL-6. Unsorted spleen cells or sorted L3T4$^+$ and Lyt-2$^+$ splenic T cell subpopulations (both 96–97% pure) were incubated with *(shaded bars)* or without *(open bars)* HP1/IL-6 (10^4 U/ml) in the presence of a submitogenic dose of PHA (1/2,000 for unsorted and L3T4$^+$ cells and 1/4,000 for Lyt-2$^+$ cells). Thymidine incorporation was measured on day 4 (mean of triplicate measurements, 1 SE).

cell growth factor activity. Evidence against this possibility was provided by the following observations: *(a)* hybridoma growth factor purified from supernatants of L cells (L-HGF), which produce no IL-4, was as active as HP1/IL-6 (Table I, Exp. 2), and *(b)* anti-IL-4 mAb 11B11, which completely inhibited the proliferative response of T cells to IL-4, had no effect on their response to HP1/IL-6 (Fig. 2). The involvement of other contaminants was ruled out by the observation that anti-HP1/IL-6 mAb 6B4 completely and specifically abolished the HP1/IL-6–induced T cell proliferation (Fig. 2).

Proliferation of Thymocytes in Response to HP1/IL-6. In the presence of PHA, HP1/IL-6 induced thymocyte proliferations that were 5–10 times stronger than those obtained with IL-1 (Table II). This observation, together with the fact that IL-1 is a potent inducer of IL-6 in human fibroblasts (9), prompted us to examine the possible involvement of HP1/IL-6 in the response of thymocytes to IL-1. Anti-HP1/IL-6 mAb 6B4, which completely blocked the proliferation of thymocytes induced by HP1/IL-6, had, however, no significant effect on the IL-1-induced proliferation (Table III), indicating that thymocyte activation by IL-1 is probably not mediated by HP1/IL-6. In this context, it is noteworthy that HP1/IL-6 was also inactive in assays for IL-1 based either on the induction

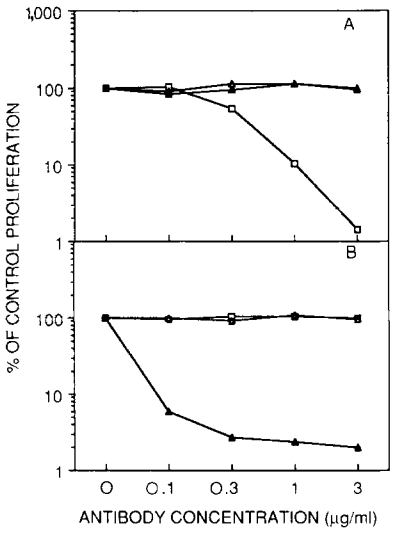

FIGURE 2. Inhibition of T cell proliferation by anti-HP1/IL-6 and anti-IL-4 mAbs. PHA-activated spleen cells were incubated with HP1/IL-6 (2×10^3 U/ml, □), IL-2 (50 U/ml, △) or IL-4 (50 U/ml, ▲), and increasing concentrations of anti-HP1/IL-6 *(A)* or anti-IL-4 *(B)* mAbs. Thymidine incorporation was measured on day 4 in triplicate cultures.

TABLE II
Proliferation of PHA-activated Thymocytes in Response to IL-1 and HP1/IL-6

Cytokines	Dose	Thymidine incorporation	Stimulation index
	U/ml	*cpm*	
IL-1	0	2,363	
	0.2	4,131	1.7
	1	8,497	3.6
	5	17,341	7.3
	25	17,444	7.4
HP1/IL-6	0	1,879	
	80	2,078	1.1
	400	7,479	4.0
	2,000	78,053	41.5
	10,000	169,606	90.3

Optimal numbers of thymocytes (1.5×10^6 cells/well for IL-1 and 0.5×10^6 cells/well for HP1/IL-6) were cultured in the presence of PHA (1/500) and of the indicated doses of cytokines. Thymidine incorporation was measured on day 3 after a 6-h pulse (mean of triplicate cultures, SE <10%).

of IL-2 secretion by T cell lymphoma LBRM-33-A15 (25) or on the induction of the response of unstimulated thymocytes to IL-2 (26) (data not shown).

Profound differences were observed in the responses of individual thymocyte subsets to HP1/IL-6. Immature $L3T4^+$,$Lyt\text{-}2^+$ cells, which proliferated significantly although more weakly than the mature cells in the presence of IL-2, were

TABLE III
Anti-HP1 6B4 mAb Inhibits HP1/IL-6– but not IL-1-induced Thymocyte Proliferation

Anti-HP1/IL6	Thymocyte proliferation (percent of control) induced by:	
	HP1/IL-6*	IL-1‡
μg/ml		
0.1	62.8	94.4
0.3	33.4	86.0
1	9.4	88.1
3	0.3	89.8
10	−1.5	90.2
30	−4.0	91.3

Proliferation of PHA-activated thymocytes was measured on day 3. Control cultures were incubated without antibody. Results were calculated as follows: 100 × [(cpm with PHA, cytokine, and antibody) - (cpm with PHA alone)]/[(cpm with PHA and cytokine) - (cpm with PHA alone)].
* 2,000 U/ml.
‡ 16 U/ml.

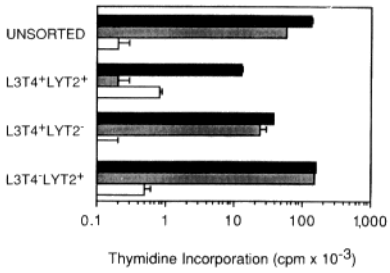

FIGURE 3. Differential response of thymocyte subpopulations to HP1/IL-6 and IL-2. Proliferation of PHA-activated thymocyte subpopulations in control *(open bars)* cultures and in cultures stimulated with HP1/IL-6 (10^4 U/ml, *shaded bars*) or IL-2 (100 U/ml, *solid bars*). Thymidine incorporation was measured on day 3 (mean of triplicate cultures, 1 SE). Sorted $L3T4^+Lyt\text{-}2^+$, $L3T4^+Lyt2^-$, $L3T4^-Lyt\text{-}2^+$ thymocytes were 95%, 98%, and 98% pure, respectively.

totally unresponsive to HP1/IL-6, whereas mature $L3T4^+$,$Lyt\text{-}2^-$ and $L3T4^-$,$Lyt\text{-}2^+$ cells responded equally well to both cytokines (Fig. 3).

Proliferation of Accessory Cell–depleted T Cells in Response to HP1/IL-6. To examine the possible involvement of accessory cells in the responses to HP1/IL-6, we purified mesenteric T cells by sequential passages over nylon-wool columns and treatment with anti-Ia antibodies and complement. The cells collected at various stages of this procedure were then stimulated, in the presence or absence of HP1/IL-6, with a concentration of Con A that yielded maximal proliferation of unfractionated cells. The population obtained after two nylon-wool passages and two anti-Ia treatments, which consisted of >98% $Thy\text{-}1^+$ cells, and contained <0.2% accessory cells by nonspecific esterase staining, was totally unresponsive to this dose of Con A, indicating a rigorous depletion of accessory cells (27, 28). As shown in Table IV, the response of these cells to Con A was almost completely restored upon addition of HP1/IL-6. Likewise, the synergistic activation of T cells by HP1/IL-6 and PHA or insolubilized anti-T3 antibodies

TABLE IV

Proliferation of Accessory Cell–depleted T Cells in Response to HP1/IL-6 and Various T Cell Stimuli

Treatment of responding cells		Costimuli	Without HP1/IL-6	With HP1/IL-6
Nylon	Anti-Ia			
			cpm	
—	—	None	463	1010
		Con A*	71,846	146,280
Once	—	Con A	37,658	143,690
Twice	—	Con A	15,720	104,397
Twice	Once	Con A	241	92,507
Twice	Twice	Con A	58	90,653
		PHA‡	449	124,320
		Anti-T3–Sepharose§	33	15,872

Mesenteric lymph node cells were fractionated by one or two passages over nylon-wool columns followed by one or two treatments with anti-Ia antibodies and complement. They were then incubated (5×10^4/well) with or without 10^3 U/ml of HP1/IL-6 in the presence of the indicated costimuli. Thymidine incorporation was measured on day 4 (mean of triplicate measurements, SE<10%).

* 2 μg/ml

‡ 1/1,000

§ 2.5 μg/ml

TABLE V
Failure of Helper T Cell Lines to Respond to HP1/IL-6

T cells	Control	Proliferation		
		IL-2	IL-4	HP1/IL-6
		cpm × 10^{-3}		
TUC2.15	3.7	195	117	5.5
TUC5.37	0.3	140	2.1	0.1
TUC7.5	0.1	172	0.3	0.1
TUC7.8	0.4	50	0.3	0.1
TUC7.19	0.1	168	0.6	0.1
TUC7.29	0.1	133	0.2	0.1
TUC7.33	0.2	142	105	0.1
TUC7.37	0.1	161	6.5	0.6
TUC7.52	0.1	56	0.1	0.2
TUC7.58	0.2	135	1.3	0.2
TUC13	1.8	133	8.9	2.2
TUC7.51 rested	0.1	81	0.4	0.1
blasts	1.2	272	17.4	0.1

T cells from 2-wk-old cultures (5 × 10^4 cells/well) were incubated for 2 d with IL-2, IL-4 (100 U/ml each), or HP1/IL-6 (10^4 U/ml) and pulsed with thymidine (0.5 μCi/well) for 6 h. Blasts were obtained by a 24-h incubation with antigen and feeder cells.

was also unaffected by accessory cell depletion (Table IV). Similar results were obtained with splenic T cells prepared by the same procedure (data not shown).

Lack of Response of Helper T Cell Lines to HP1/IL-6. The proliferation of several helper T cell lines in the presence of HP1/IL-6 was compared with the

TABLE VI
Stimulation by HP1/IL-6 and IL-2 of Cytolytic T Cell Responses In Vitro

Exp.	Cytokine	Recovery	Lytic activity on	
			P815	EL4
		%		
1	None	53	10	<10
	HP1/IL6	78	814	<10
	IL2	100	2,491	53
2	None	50	8	NT
	HP1/IL6	90	1,140	NT
	IL2	145	6,300	NT

C57BL/6 spleen cells (2 × 10^6) were stimulated with 2 × 10^6 irradiated DBA/2 high-density spleen cells in the presence of HP1/IL-6 (10^4 U/ml) or IL-2 (100 U/ml) as described in Materials and Methods. Cytolytic activity was measured on day 6 using 2,000 ^{51}Cr-labeled P815 or EL4 target cells. Results are expressed in lytic units per culture, one unit corresponding to the cytolytic activity required to obtain 20% specific lysis. NT, not tested.

responses obtained with IL-2 and IL-4 (Table V). In the absence of any other stimulus, the 12 lines tested proliferated extensively in response to IL-2, but none responded to HP1/IL-6. The responses to IL-4 were intermediate, with about half the lines proliferating significantly and two showing extensive proliferation. Costimulation with PHA or the use of blastic rather than resting cells increased the responses to IL-4 but failed to induce any response to HP1/IL-6.

Stimulation of Cytolytic T Cell Differentiation by HP1/IL-6. The T cell responses to HP1/IL-6 examined so far were exclusively of proliferative nature. To evaluate the ability of HP1/IL-6 to also induce T cell differentiation, we tested its influence on the development of a primary in vitro cytolytic T cell response. As shown in Table VI, HP1/IL-6 enhanced the primary anti-H-2^d cytolytic response of C57BL/6 spleen cells ~100-fold with little effect on cell recoveries. These responses were extremely specific, indicating that HP1/IL-6 stimulated the differentiation of only those CTL precursors that had been activated by a specific interaction with antigen.

Discussion

HP1/IL-6, the murine hybridoma and plasmacytoma growth factor, was found to stimulate the proliferation of mature thymic and peripheral T cells in the presence of submitogenic doses of either PHA or anti–T cell receptor antibodies. Both the $L3T4^+$ and the Lyt-2^+ T cell subsets responded to this stimulation, which was completely inhibitable by an anti-HP1/IL-6 mAb and did apparently not require accessory cells. In addition, primary allogeneic cytolytic T cell responses were also enhanced in the presence of HP1/IL-6.

Half-maximal proliferation of mature T cells was induced with ~100 pg/ml of HP1/IL-6, which is well within the physiological range, since titers equivalent to 100 ng/ml have been detected in the serum of mice injected with endotoxin (29). This concentration is ~10 times lower than that required for half-maximal stimulation of normal B cells (14), which suggests that in spite of the original identification of HP1/IL-6 as a growth factor for transformed B cells, the primary immunological target of this molecule may be the T rather than the B lymphocyte. The observation that B cells, unlike T cells, require costimulation with IL-1 to become fully responsive to HP1/IL-6 (14) would also seem to support this idea.

T cell activation by lectins requires the presence of accessory cells (27, 28). The observation that HP1/IL-6 could restore the response of accessory cell–depleted T cells to Con A suggests that production of this molecule by macrophages (3, 29) plays a significant role in the ability of these cells to support such responses, and raises the question of the relation between HP1/IL-6 and recently described accessory cell–replacing factors (30, 31). The mechanisms involved in T cell activation by HP1/IL-6 have not yet been fully clarified. Preliminary experiments using anti-IL-2-R antibodies suggest that HP1/IL-6 could operate by inducing the production of IL-2 (manuscript in preparation). However, we have not been able so far to detect any IL-2 in the supernatant of HP1/IL-6–activated T cells.

The response of PHA-activated peripheral T cells to HP1/IL-6 was quantita-

tively similar to that obtained with IL-2 and usually better than that induced by IL-4. Several observations, however, indicate that T cells are less uniformly responsive to HP1/IL-6 than to IL-2. Indeed, certain T cell subsets, such as immature thymocytes, were totally unresponsive to HP1/IL-6 under conditions where they significantly responded to IL-2. Moreover, all our helper T cell clones or lines failed to proliferate in the presence of HP1/IL-6, suggesting that cells repeatedly activated by exposure to antigen, which in our hands become increasingly responsive to IL-2 and IL-4, lose their responsiveness to HP1/IL-6. This observation suggests that, unlike IL-2, HP1/IL-6 may primarily act during the initial stages of T cell activation, which would be consistent with the finding that the number of IL-6 receptors is higher on resting than on activated human T cells (32). Considering the wide variety of cells both within (e.g., macrophages) and outside (e.g., fibroblasts) the immune system (3, 4) that can be induced to produce HP1/IL-6 by viral infections or exposure to endotoxin, it is tempting therefore to speculate that this molecule could play a unique role in initiating T cell responses by enabling cells that do not belong to the immune system to activate T cells directly.

Summary

Interleukin-HP1 (HP1)/IL-6 is a 25–30-kD protein produced by macrophages, fibroblasts, and certain T cell lines. It was originally identified as a mouse growth factor for B cell hybridomas and plasmacytomas, and was recently shown to stimulate growth and differentiation of normal B cells. Here we demonstrate that, in the presence of lectins or anti–T cell receptor antibodies, HP1/IL-6 has a growth factor activity equivalent to that of IL-2 for mature thymic and peripheral T cells of both the $L3T4^+$ and $Lyt\text{-}2^+$ subsets. Contrary to IL-2 and IL-4, HP1/IL-6 was, however, not capable of supporting the growth of established T cell lines. In addition to its effects on T cell proliferation, HP1/IL-6 also enhanced the differentiation of mouse cytolytic T cell precursors in primary allogeneic mixed lymphocyte cultures.

Fractionation of responding cell populations indicated that HP1/IL-6 was capable of restoring the response of accessory cell–depleted T cells to Con A. This observation suggests that the production of HP1/IL-6 by macrophages could, at least partly, explain their role in polyclonal T cell activation.

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Note added in proof: Garman et al. (33) have recently shown that human IL-6 induces proliferation of mouse T cells and that this proliferation can be inhibited by anti-IL-2-R antibodies.

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