Clin Exp Immunol 1993; 93:471–478

Interaction of IL-1β, IL-6 and tumour necrosis factor-alpha (TNF-α) in human T cells activated by murine antigens

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(Accepted for publication 26 May 1993)

SUMMARY

We used a mixed leucocyte culture between human T cells and irradiated murine splenocytes which allowed us to distinguish between cytokine production from the responder and stimulator cells by the use of species-specific assays for mRNA up-regulation. Using this model of T cell activation by antigen, we studied the effects of human antigen-presenting cell-derived cytokines IL-1β, IL-6 and TNF-α on the activation of human T cell subsets. We show in this system that exogenously added IL-1β, IL-6 and TNF-α induces IL-2 receptor (R) up-regulation and IL-2 production, and proliferation by both $CD4^+$ and $CD8^+$ cells. The addition of IL-1β induces IL-6 mRNA, and anti-IL-1 antibodies or an IL-1R antagonist protein completely suppresses IL-6 and TNF-α supported proliferation. Similarily, addition of IL-6 or TNF-α induces up-regulation of IL-1β mRNA. However, anti-IL-6 and anti-IL-6R antibodies only partially block proliferation supported by IL-1β. These findings suggest that IL-6 and TNF-α will induce IL-2R up-regulation/IL-2 secretion via the induction of IL-1β production.

Keywords cytokine interaction T cell activation

INTRODUCTION

It has been shown that human T cells require secondary signals, such as antigen-presenting cell (APC)-derived cytokines IL-1, IL-6 and TNF-α to proliferate [1–9]. These cytokines can have additive effects with each other [6,9–11]; other reports proposed that a combination of IL-1 with either IL-6 or TNF-α is required for optimal T cell activation [12–14]. These findings suggest that certain T cell subsets require particular cytokines, which may not be essential for other T cells [15]. Moreover, cytokines may be regulated interdependently [11,13] and IL-1, IL-6 and TNF-α are each capable of inducing their own production (for review see [16,17]). These previous studies were performed on APC- or mitogen-activated T cells. The response of T cells to antigen in a mixed leucocyte culture (MLC) more closely mimics *in vivo* response than mitogen-induced activation, however. So far, the role of APC-derived cytokines produced by human T cells which are responding to antigen has not been examined.

Human T cells proliferate in response to alloantigen in an MLC in the presence of responder or stimulator APC [18–20]. However, in a xenogeneic MLC between human T cells and irradiated murine splenocytes proliferation will only occur in the presence of responder APC [21]. Previous findings indicate that recombinant human (rh) IL-1β or IL-2 can serve as a secondary signal and thus substitute for APC in order to achieve T cell proliferation in response to murine splenocytes [22,23]. This model provides us with the opportunity to study the effects of human cytokines on antigen-stimulated human T cells. Using species-specific assays for mRNA we were able to distiguish between cytokines from the responder and stimulator cells, and thus to determine the interaction of cytokines in the responding T cells. We studied whether the activity of IL-1, IL-6 and TNF-α activated different T cell subpopulations, and whether the exogenous addition of one cytokine would lead to the production of another one by the activated T cells.

For the first time in an antigen-presenting setting we can show that all three recombinant human cytokines (IL-1β, IL-6 and TNF-α) can provide a secondary signal for both $CD4^+$ and $CD8^+$ cells. Both rhIL-6 and rhTNF-α can enhance the effects of rhIL-1β. Our data support a concept that by coculturing human T cells with murine splenocytes, IL-6 and TNF-α will induce IL-2R up-regulation/IL-2 secretion via the induction of IL-1β production.

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MATERIALS AND METHODS

Reagents

Unconjugated MoAbs Mo2 and NKH-1A were purchased from Coulter Immunology (Hialeah, FL); MoAbs OKT3, OKT4 and OKT8 were obtained from Ortho Diagnostic System (Raritan, NJ); MoAbs Leu-7, Leu-11b, DR and FITC- or PE-conjugated

MoAbs Leu-4 (anti-CD3), Leu-3a (anti-CD4), Leu-2a (anti-CD8), Leu-7, Leu-M5, Leu-11c (anti-CD16), Leu-19 (anti-CD56), and anti-IL-2R (p55, CD25) were purchased from Becton Dickinson (Mountain View, CA). Anti-TAC (CD25 [24]) was a generous gift of Dr T. Waldman (National Institute of Health, Bethesda, MD). Polyclonal goat anti-human IL-6 and MoAb PM1 (final concentrations 1:1000 and 1 μg/ml, respectively) were kindly provided by Drs T. Kishimoto, T. Hirano, and T. Taga (Osaka University, Osaka, Japan). Polyclonal rabbit anti-human IL-1β (final concentration 32 μl/ml) and MoAb anti-human IL-1R (final concentration 1·2 μl/ml) were from Genzyme (Boston, MA); control antibodies were matched for species and used separately or in combinations, when appropriate. IL-1 receptor antagonist protein (IRAP [25]) was a generous gift from the Upjohn Company (Kalamazoo, MI) and used at a final concentration of 15·6 μg/ml. Recombinant hIL-1β was purchased from Genzyme (1 U = 5 pg), rhTNF-α (1 U = 5000 pg) was from R&D Systems (Minneapolis, MN); rhIL-6 was a gift from Ajinomoto Co., Inc. (Kawasaki, Japan; 1 U = 400 pg). Phytohaemagglutinin (PHA)-P (Sigma, St Louis, MO) was used at 10 μg/ml.

Cell preparation

Human APC were prepared and irradiated with 35 Gy; T cells and $CD4^+$ and $CD8^+$ subpopulations were purified as described previously [26]. The resulting T cells were 97–99% $CD3^+$ ($CD4^+$ 60–72%, $CD8^+$ 15–40%, $CD4^+CD8^+$ 1–3%, $CD4^-CD8^-$ < 1%). Less than 1% of cells were Leu-19^+; no reactivity was detectable with MoAbs Leu-7, anti-DR, Leu-M5 or Leu-11c. The proliferative response of purified T cells to PHA was median 1·9%, range 0·4–3·2%, of the response of these cells reconstituted with 20% irradiated APC. CD8-depleted cells were 91–98% $CD4^+CD8^-$ and CD4-depleted cells were 85–93% $CD4^-CD8^+$ (five and three independent experiments, respectively).

Murine spleen cells were stimulating cells from BALB/c mice irradiated with 25 Gy.

Mixed leucocyte cultures

Responding human T cells (1×10^5) and stimulating murine splenocytes (1×10^6) were seeded in triplicates into flat-bottomed 96-well plates (Linbro, Flow Laboratories, MacLean, VA). Cytokines or tissue culture medium (TCM) were added to a total volume of 0·2 ml. Positive control cultures contained 5–10% autologous APC. Proliferation was tested by ^{3}H-TdR incorporation.

Detection of IL-2 in vitro *culture supernatants*

Culture supernatants were collected after 72 h, 96 h, 120 h and 135 h of culture and assayed for their ability to induce proliferation of CTLL-2 [27] cells as described previously [26]. In a separate experiment this (sub)cell line did not respond proliferatively to rhIL-4.

Immunofluorescence analysis

Two to five $\times 10^5$ cells were stained with FITC- or PE-conjugated MoAb in V-bottomed 96-well plates. Unfixed cells were evaluated by flow cytometry, using a FACStar Plus (Becton Dickinson). Dead cells were excluded on the basis of forward light scatter. Each experiment included staining with isotype-matched control mouse immunoglobulin.

Message amplification phenotyping [28]

RNA was extracted by the method of Chomczynski & Sacchi [29] from $1·5–3 \times 10^6$ human cells. Synthesis of cDNA essentially was performed as described [28]. Oligomers for human and murine IL-1β, IL-2, IL-2R, IL-6 and β-actin were from Clontech (Clontech Laboratories Inc., Palo Alto, CA), and polymerase chain reaction (PCR) assays were performed as suggested by the manufacturer. After PCR amplification (30–35 cycles), the samples were run on 5% polyacrylamide gels. For each sample PCR analysis was performed at least twice. In some cases, agarose gels were prepared and analysed by Southern hybridization using template ^{32}P-labelled DNA corresponding to the amplified cytokine product. In a set of two experiments we tested whether human oligomers would also react with murine cDNA of the respective murine cytokine. No such cross-species amplification was detectable for any of the cytokines/cytokine receptors studied. We tested whether contamination with human APC in the purified T cell population would bias the PCR-derived results. Freshly isolated purified human T cells were reconstituted with 0·5% or more APC, cultured for 2 h and then used for the PCR assay. Messenger RNA for IL-1β was only detectable in samples reconstituted with 1% or more of APC. This reconstituted T cell population, but not T cells mixed with less APC, also responded proliferatively to PHA. Thus, an effect of APC contamination on PCR was controlled by PHA responsiveness.

RESULTS

T cell subpopulation responding to mouse splenocytes

Previous studies have indicated that in mice only $L4T8^+$ cells express IL-1R [30], and we have shown that in humans only $CD4^+$ T cells will respond to rhIL-1β, if activated with cross-linked OKT3 [26]. We were therefore interested in investigating the responsiveness of separated $CD4^+$ and $CD8^+$ cells to murine antigens in the presence of rhIL-1β. As shown in Fig. 1, both T cell subpopulations proliferated in response to murine splenocytes if cultures contained rhIL-1β (representative for eight independent experiments).

Whether $CD4^+$ or $CD8^+$ cells are the predominant responders to rhIL-6 is still disputed [31–33]. We therefore examined the proliferative response of separated $CD4^+$ and $CD8^+$ cells in the presence of rhIL-6 with or without rhIL-1β. We tested whether rhIL-6 alone acts on both subsets, and whether the combination of rhIL-6 with rhIL-1β activated cells distinct from those activated by rhIL-1β alone. As shown in Fig. 1a, separated $CD4^+$ and $CD8^+$ cells proliferated in response to mouse splenocytes if rhIL-1β and/or rhIL-6, or APC (as a positive control) were added to the cultures. The response seen with rhIL-1β was enhanced by the addition of rhIL-6 in panT cells as well as in both separated T cell subpopulations (eight independent experiments). Thus, rIL-1β acted on $CD4^+$ and $CD8^+$ cells, and the augmentation of proliferation induced by rhIL-6 was through the activation of additional cells in both T cell subpopulations.

Again, we tested whether the augmentive effects of rhTNF-α on T cell proliferation induced by rhIL-1β was due to activation of a particular T cell subset. rhTNF-α served as a secondary signal for separated $CD4^+$ and $CD8^+$ cells stimulated with murine splenocytes (Fig. 1b); however, an enhancement of proliferation of panT cells (10/14 experiments) but not of T cell

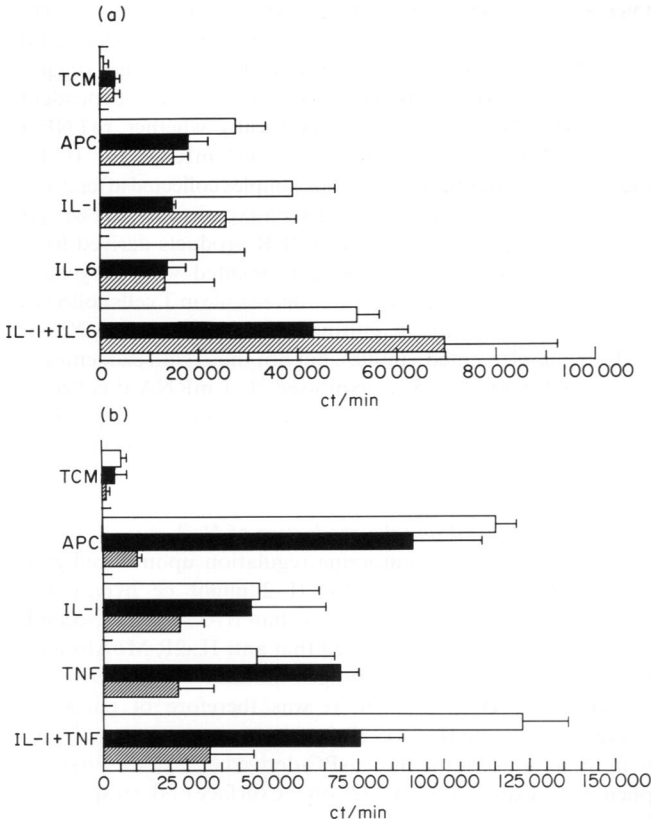

Fig. 1. Proliferative response of purified panT cells (□), and separated $CD4^+$ (■) and $CD8^+$ cells (■) to murine splenocytes in the presence or absence of autologous responder antigen-presenting cells (APC), or rhIL-1β, or rhIL-6, or rhTNF-α. Cells were cultured with murine splenocytes for 7 days. ^{3}H-TdR was added to the cultures for the last 8 h of culture. Bars indicate s.d. TCM, Tissue culture medium.

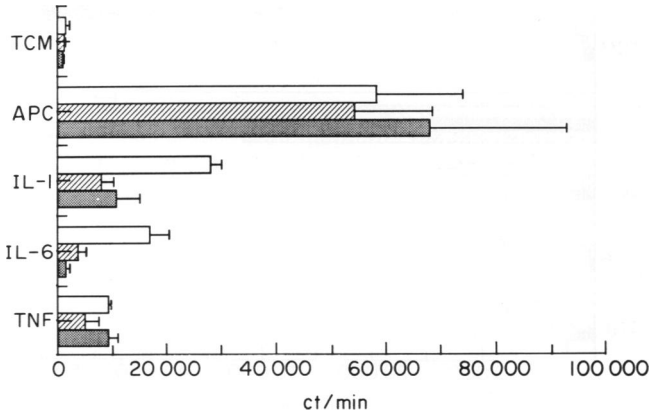

Fig. 2. Inhibitory effects of polyclonal anti-IL-6 (■) and anti-IL-6R MoAb (▨) on the proliferative response of purified human T cells stimulated with murine splenocytes. Rabbit immunoglobulin and murine ascites served as controls (□). Experimental design as in Fig. 1. TCM, Tissue culture medium.

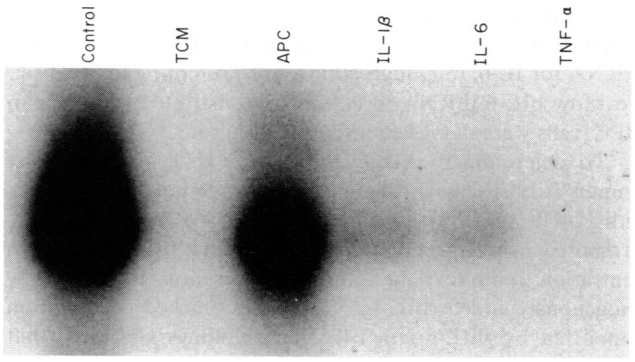

Fig. 3. Up-regulation of IL-6 mRNA in human T cells stimulated with murine antigen. Human T cells were cultured together with murine splenocytes; samples with responder antigen-presenting cells (APC) were collected after 2 h, those from all other experimental conditions were collected after 42 h of culture for the determination of mRNA for human IL-6 (see Materials and Methods). The proliferative response (^{3}H-TdR incorporation) after 7 days of culture was: TCM 1430 ± 411 ct/min, APC 58 436 ± 15 630 ct/min, rhIL-1β 28 028 ± 2185 ct/min, rhIL-6 16 926 ± 3622 ct/min, rhTNF-α 9383 ± 411 ct/min. TCM, Tissue culture medium.

subpopulations (two independent experiments) was seen if rhTNF-α was added to cultures supplemented with rhIL-1β. The results were similar if proliferation was evaluated 1 or 2 days earlier, excluding a possibility that the combination of cytokines had induced an earlier peak of proliferation in panT cells than in T cell subsets. Thus, the augmentive effects of rhTNF-α on the proliferative response promoted by rhIL-1β on panT cells may have needed collaboration between $CD4^+$ and $CD8^+$ cells. Alternatively, $CD4^+$ cells may have been maximally stimulated by rhTNF-α alone, while TNF-α-activated $CD8^+$ cells may have become self-suppressive under the influence of rhIL-1β.

Interaction of APC-derived cytokines in the process of T cell activation

First, we addressed the dependency of the activity of IL-1β in supporting T cell activation on the presence of IL-6 and *vice versa* by using antibodies specific for the respective cytokine. As shown in Fig. 2 (representative of three independent experiments), we observed that anti-IL-6 or anti-IL-6R, at a concentration inhibitory to proliferation induced by exogenously added rhIL-6, decreased the response promoted by rhIL-1β by about 50%. This observation suggests that IL-1β induces IL-6 production, and that both IL-1β and IL-6 are acting in concert on human T cells. We pursued this hypothesis by evaluating mRNA for IL-6 in cultures supplemented with IL-1β. IL-6 mRNA in monocytes peaks 4 h after activation, while T cells up-regulate IL-6 mRNA 40–48 h after induction of stimulation [34]. This difference assisted us in assigning the detected IL-6 mRNA to T cells rather than to APC possibly contaminating the purified T cell population. Using the standard message amplification phenotyping (MAPPing) procedure [28] a band corresponding to the expected IL-6 PCR product was seen, along with other non-specific bands. To confirm the specificity of these findings, PCR products were run on agarose gels, and analysed by Southern hybridization using template ^{32}P-labelled cDNA corresponding to human IL-6 (provided by Clontech). As shown in Fig. 3, mRNA for hIL-6 became detectable if cultures had been supplemented with IL-1β (cells harvested after 42 h of

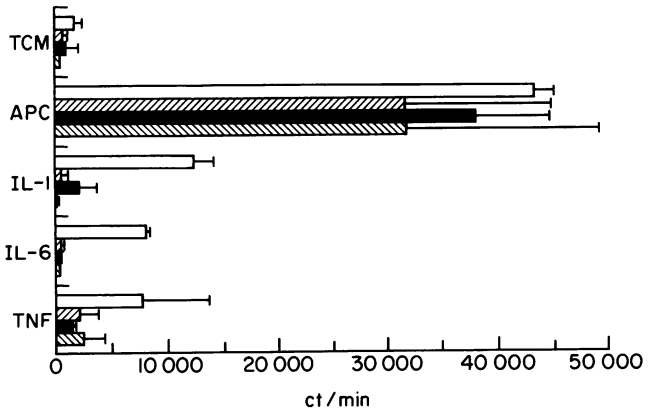

Fig. 4. Inhibitory effects of polyclonal anti-IL-1 (■), MoAb anti-IL-1R (■), and IL-1 receptor antagonist protein (IRAP) (▧) on the proliferative response of purified human T cells stimulated with murine splenocytes. Goat immunoglobulin and murine ascites served as controls (□). Experimental design as in Fig. 1. TCM, Tissue culture medium.

culture). No such message was detectable if rhTNF-α was added to the cultures, even though T cells were actively proliferating. It is of note that the exogenous addition of rhIL-6 also resulted in mRNA for IL-6, thus suggesting a self-promoting mechanism. To show hIL-6 mRNA in cultures reconstituted with human APC, cells were harvested after 2 h of culture.

To determine if rhIL-6 will induce IL-1 production by human T cells we next examined whether or not anti-IL-1β, or anti-IL-1R, or IRAP would block the proliferative response promoted by rhIL-6. Both antagonists and IRAP, at a concentration inhibitory to T cell proliferation promoted by exogenously added rhIL-1β, completely blocked proliferation supported by rhIL-6 (Fig. 4). These findings suggested that supplementation of cultures with rhIL-6 had induced IL-1 production. Neither anti-IL-1β, anti-IL-1R, nor IRAP significantly reduced the proliferative response of T cells supplemented with autologous APC. We therefore assume that APC were able to bypass the need for IL-1β for the activation of the T cells, or that cultures with APC contained more IL-1 than could be blocked by the anti-IL-1 reagents at the concentration that had been added.

We more critically tested our conclusion, that upon the addition of rhIL-6, T cells produce IL-1 by evaluating the presence of mRNA for IL-1β in the responder cells. The hIL-1β-specific PCR products derived from T cells stimulated with antigen plus rhIL-6 were tested further for their specificity by Southern hybridization. As shown in Fig. 5, mRNA for IL-1β was detectable in samples derived from cultures supplemented with rhIL-6, thus suggesting that IL-6 can stimulate IL-1β production in human T cells.

We hypothesized that TNF-α can induce IL-1 production in T cells (as it does in monocytes [17]), which can then serve as a costimulatory signal; if rhTNF-α acts only via IL-1 production, adding rhTNF-α to the cultures would not have any further effects than those induced by rhIL-1β, which has already been in the cultures at an optimal concentration. However, we have demonstrated that rhTNF-α augmented rhIL-1β-induced T cell proliferation in most cases (Fig. 1b). To address the question whether the activity of rhTNF-α is only via IL-1 production, or if rhTNF-α has intrinsic effects on T cells activated by murine splenocytes, we used anti-IL-1 antibodies or blocking of the IL-1 receptor. As shown in Fig. 4, all anti-IL-1 reagents decreased proliferation supported by rhTNF-α. These findings suggest that rhTNF-α acted on the T cells via an IL-1-dependent mechanism. To address more specifically whether rhTNF-α induced IL-1 production we determined mRNA for IL-1β. Figure 5 illustrates the results from samples collected after 2 and 9 h of culture from one out of four such experiments. Messenger RNA for IL-1β was detectable in PCR products derived from 2-h cultures, which had been supplemented with exogenous rhTNF-α. No mRNA for IL-1 was detectable in T cells collected from cultures containing murine splenocytes only. APC- or rhIL-1β-supplemented samples, but not those with exogenously added rhIL-6 or rhTNF-α, expressed IL-1 mRNA if collected after 9 h of culture. These results are in line with a previous study, indicating differential control of the expression of the IL-1β gene [35].

Expression of IL-2R and the production of IL-2

IL-2/IL-2R undergoes autocrine regulation upon T cell activation. However, the need for IL-2 might be bypassed if exogenously added cytokines other than IL-2 served as secondary signals. It has been proposed that anti-IL-2R MoAbs have little inhibitory effect on mitogen-induced T cell activation supported by IL-6 [3–5,10]. It was therefore of particular interest to evaluate IL-2/IL-2R involvement in T cells activated with the support of the three APC-derived cytokines. First, the phenotypic expression of CD25 on the surface of the responding T cells was analysed 5 days after initiation of cultures. Table 1 shows results from one experiment (representative of five independent experiments). T cells in culture containing human APC or cytokines expressed CD25. The number of $CD25^+$ cells increased if rhIL-1β was combined with rhIL-6 or rhTNF-α. Mean channel fluorescence, as a measure of CD25 density on the cell surface, shifted to the right if rhIL-1β was used in combination with rhIL-6 or rhTNF-α, compared with either cytokine alone.

To test whether coculturing human T cells with murine antigens but with neither human APC nor cytokines would already induce signalling to T cells, though without CD25 up-regulation, we determined mRNA for IL-2R. In all experimental conditions containing T cells together with murine splenocytes, mRNA for IL-2R was detectable as early as 2 h after initiation of cultures; message for IL-2R was still detectable in samples collected after 20 h of culture (data not shown). These findings indicate that cells became activated already by the addition of murine antigens, and further functional maturation was supported by cytokines.

In view of the current controversy as to whether or not T cell activation supported by IL-6 can be inhibited by anti-CD25 MoAb [3–5,32,36], we evaluated the blocking activity of this MoAb on human T cells responsive to murine antigens. The results from five independent experiments indicate that anti-CD25 MoAb, at a final concentration of 2×10^{-4}, inhibited proliferation in all experimental conditions, except in control cultures containing responder APC (Fig. 6). The role of IL-2/IL-2R was further investigated in two other independent experiments, with the use of anti-IL-1 reagents together with anti-IL-6 antibodies. In these experiments neither the combination of anti-IL-1 together with anti-IL-6 antibodies, nor of anti-IL-1R or IRAP together with anti-IL-6R, inhibited T cell proliferation

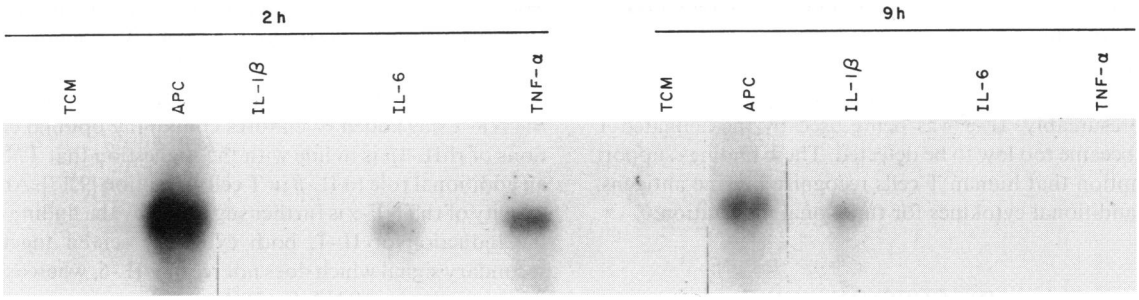

Fig. 5. Up-regulation of IL-1β mRNA in human T cells from Fig. 4. Human T cells were cultured together with murine splenocytes for 2 h and 9 h, when samples were collected for the determination of mRNA for human IL-1β. Autoradiograms from antigen-presenting cell (APC)-supplemented polymerase chain reaction (PCR)-derived samples were exposed for 8 h, those from all other experimental conditions for 24 h. TCM, Tissue culture medium.

Table 1. Activation of purified human T cells by murine antigens in the presence of autologous antigen-presenting cells (APC) or various recombinant human cytokines. Up-regulation of CD25 and concentration of IL-2 in culture supernatants.

Culture condition	^{3}H-TdR incorporation (ct/min)	CD25$^+$ cells (%)	Mean channel on log scale	IL-2 concentration in supernatants (pg/ml)
T TCM	52 ± 10	0	505	< 100
T Mx	741 ± 135	1	527	133
T Mx + APC	47 833 ± 5095	8	618	1998
T Mx + IL-1	23 024 ± 4377	10	638	243
T Mx + IL-6	4077 ± 1782	6	555	233
T Mx + TNF-α	15 086 ± 657	8	604	243
T Mx + IL-1 + IL-6	40 688 ± 16 004	20	648	1032
T Mx + IL-1 + TNF-α	41 475 ± 7406	14	660	1019

Purified human T cells were cultured with mouse splenocytes for 7 days when proliferation was evaluated. Expression of CD25 and mean channel fluorescence were determined from 20 000 events from each experimental combination after 5 days of culture. Supernatants were collected after 3 days of culture for the determination of IL-2 (see Materials and Methods).

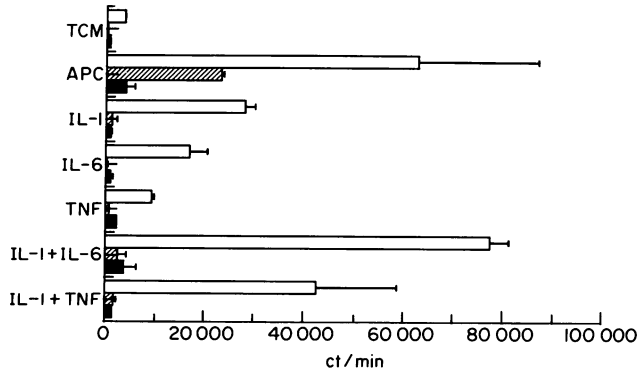

Fig. 6. Inhibitory effects of anti-IL-2R (anti-CD25) on the proliferative response of purified human T cells stimulated with murine splenocytes. Cells were cultured either with control ascites or with anti-CD25 at a final concentration of 10^{-4} or 2×10^{-4}. Experimental design as in Fig. 1. □, Control; ■, anti-CD25 2×10^{-4}; ■, anti-CD25 10^{-4}. TCM, Tissue culture medium.

in the presence of autologous APC. However, combining the latter antibodies (with or without IRAP) with anti-CD25 MoAb completely blocked proliferation, substantiating the critical role of IL-2R (data not shown). A two-fold higher concentration of anti-CD25 MoAb was also inhibitory, even without any further blocking reagents. Thus, activation of the human T cell with murine spleen cells and either APC or cytokines, including rhIL-6, depends mainly on IL-2R occupancy.

We hypothesized that the three APC-derived cytokines regulate IL-2 production by the T cells differently. To test this hypothesis, we measured the IL-2 concentration in culture supernatants from five independent experiments after 3 days of culture. T cells were cultured together with splenocytes under various conditions in the presence of anti-CD25 MoAb to block utilization of the IL-2 produced by the T cells. As shown in Table 1, IL-2 was detectable in all experimental conditions containing mouse splenocytes. The amount of IL-2 increased with the addition of cytokines to the primary cultures; the combination of rhIL-1β with either rhIL-6 or with rhTNF-α led to an additional increase of IL-2 production. Cultures containing responder APC produced about 10-fold more IL-2 than those supplemented with a single cytokine, and even produced

twice as much IL-2 as cultures containing a combination of cytokines. At later time points, or if cultures had not been blocked with anti-CD25 MoAb, IL-2 was only detectable if cultures had been reconstituted with responder APC (data not shown); presumably, IL-2 was being used by the activated T cells, and became too low to be detected. These findings support the assumption that human T cells recognize murine antigens, but need additional cytokines for functional maturation.

DISCUSSION

We addressed the role of APC-derived cytokines on antigen-induced T cell activation. These studies are of physiologic importance, as they are closely related to *in vivo* activation of T cells. Using the xenogeneic MLC we were able to control responses brought about by each of the exogenously added cytokines. We showed that purified human T cells became proliferative in response to mouse splenocytes if APC-derived human recombinant cytokines were added to the cultures. Separated T cell subpopulations of $CD4^+$ and $CD8^+$ cells responded to stimulation with murine antigen, provided responder APC, or rhIL-1, rhIL-6 or rhTNF-α, were present. For the first time, we can demonstrate the specific interaction of the three cytokines on unprimed human T cells responding to antigen. Upon the addition of IL-1β to the cultures, mRNA for IL-6 was induced. Our studies suggest a model in which the activity of rhIL-6 and rhTNF-α on panT cells is through the induction of IL-1. It is presumed that, via the induction of endogenous IL-1, this cytokine will induce IL-2R up-regulation/IL-2 production.

Unexpectedly, rhIL-1 did serve as a costimulatory factor for both purified $CD4^+$ and $CD8^+$ T cells. It has previously been shown in mice that only L3T4 cells have receptors for IL-1 [30], and that in humans IL-1 is a costimulatory factor for $CD4^+$ cells only [26]. The mechanism by which IL-1 serves as a costimulatory factor for separated $CD4^+$ cells as well as for $CD8^+$ cells remains unclear. It has been shown that $CD8^+$ cells require IL-6 as a costimulatory factor [13,33]. We were able to demonstrate that the addition of IL-1β led to up-regulation of mRNA for IL-6 production. It might have been IL-6 generated in this way that allowed activation of $CD8^+$ cells. Unfortunately, we were not able to assay specifically for human T cell-derived protein production, since sensitive bioassays require the use of murine cells, which do not distinguish between human and murine cytokines. Low quantities of murine IL-6 were found to be already produced by murine splenocytes when seeded into cultures. This disadvantage prompted us to use mRNA detection and blocking of cytokines and receptors to estimate the effects of added cytokines. Our data indicate that IL-1 acts in concert with IL-6 to activate T cells, and that proliferation depends on functional IL-2/IL-2R. The findings that anti-IL-6 reagents did not completely abrogate the effects of rhIL-1β suggest additional IL-6 independence, or, that the anti-IL-6 reagents used were at a concentration just sufficient to suppress the effects of exogenously added rhIL-6, but not those induced by added rhIL-1β.

IL-1β and TNF-α share many biological activities (for review see [16,17,37]). *In vivo* and *in vitro* monocytes are induced by TNF-α to produce IL-1 [17], and it is believed that many of the biological effects of TNF-α are IL-1-mediated. We specifically addressed the question, whether or not rhTNF-α led to IL-1 production by the responder T cells. We demonstrated that human T cells can be induced by TNF-α to up-regulate mRNA for IL-1β. The finding that proliferation was still enhanced if rhTNF-α was added to cultures containing optimal concentrations of rhIL-1β is in line with the suggestion that TNF-α plays an additional role to IL-β in T cell activation [9,17]. An intrinsic activity of rhTNF-α is further suggested by the finding that after the induction of IL-1, both cytokines served together as a secondary signal which does not require IL-6, whereas IL-1β on its own induces mRNA for IL-6.

We have assessed the critical role of IL-2/IL-2R in the process of T cell activation by murine spenocytes studying IL-2 production, blocking of the IL-2R, and cell surface expression of CD25. All three measures demonstrate a similar pattern as far as the role of IL-2/IL-2R is concerned. In concert with previous reports [6,9,11,31], we showed that the combination of rhIL-1β with either rhIL-6 or rhTNF-α leads to an increase of the numbers of IL-2R-expressing cells and to an increase of density of this antigen on the cell surface. By RNA-PCR analysis we found that mRNA for IL-2R became up-regulated even if T cells were cultured only with murine splenocytes, even in the absence of autologous APC or exogenously added cytokines. Thus, these cells can be regarded as poised to receive help, such as might be provided by the exogenous addition or endogenous production of cytokines which allow further maturation to effector T cells [15]. Such an additional signal was then provided by either of the three APC-derived cytokines, eventually leading to IL-2 production. Our assay to measure IL-2 does not distinguish between murine and human IL-2 (or IL-4). It is possible that murine cells cocultured with human T cells produce some IL-2 and that this murine IL-2 intitiates human T cell activation. As an example, murine APC-derived IL-1 effectively induces human T cell activation [38]. In contrast, studies in our laboratory demonstrate that exogenously added murine IL-2 has very little effect on human T cells responding to murine APC. Thus, a major interaction of endogenously produced murine IL-2 with exogenously added human cytokines seems unlikely.

Autologous APC, however, may support T cell activation by a mechanism independent of IL-1R or IL-6R occupancy. Many other known molecules (e.g. chemokines) can also affect T cell activation and their subtle interaction. We focused on the three important APC-derived cytokines, and suggest that in the presence of human APC abundant cytokines were produced to compete with and displace the receptor blocking reagents at the concentrations used in our experiments. This assumption is supported by our findings that cytokine mRNA signals from cultures containing human APC were very strong, and that APC-supported proliferation was inhibited by anti-IL-1R and anti-IL-6R combined with anti-CD25.

We cannot exclude the possibility that all three cytokines were acting on the murine APC by improving their stimulatory potency rather than directly on the human T cells, and that these activated murine APC led to human T cell activation. If the activity of the cytokines was only to 'improve', in some way, the murine APC, rather than to have a direct influence on the human T cells, one would expect to see a uniform pattern of mRNA up-regulation in all experimental conditions. We demonstrated, however, that, for example, rhIL-1β-containing cultures, but not those supplemented with rhTNF-α, induced

IL-6 mRNA, or that the kinetics of mRNA for IL-1 varied depending on the exogenously added cytokine. These data suggest that the added cytokines had a specific activity on the human T cells. Alternatively, T cells may have constitutively produced low amounts of cytokines, enhancing the effects of added cytokines. However, their cytokine mRNA would be even below the detection level of the PCR-RNA analysis, as T cells cultured with murine splenocytes alone did not show IL-1 or IL-6 mRNA activity.

We have shown that the APC-derived cytokines IL-1β, IL-6 and TNF-α are costimulatory for human panT cells and $CD4^+$ and $CD8^+$ cells responding to murine antigen. Both rhIL-6 and rhTNF-α can augment the costimulatory effects of rhIL-1β. Recombinant rhIL-1β leads to IL-6 production, while both rhIL-6 and rhTNF-α induce up-regulation of mRNA of IL-1β. These data confirm and extend previous findings on the contingent series of gene activations in human T cells that bring about proliferation and immunologic function [39]. The combined activity of two or more cytokines, be it due to autocrine production induced by an exogenously added recombinant cytokine or to the provision of a combination of cytokines, eventually leads to IL-2/IL-2R-dependent proliferation.

ACKNOWLEDGMENTS

The authors are thankful to Dr Eva Renate Grümayer for technical advice and Dr Wolfgang Holter for critically reviewing the manuscript.

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