Immunology 1988 **64** 433–438

Two distinct signals regulate induction of IL-2 responsiveness in CD8$^+$ murine T cells

H. WAGNER & K. HEEG *Department of Medical Microbiology and Immunology, University of Ulm, Ulm, FRG*

Accepted for publication 4 March 1988

SUMMARY

In the model system used here, cross-linking of T-cell receptor structures (TCR) by antigen-presenting cells (APCs) is substituted by the use of anti-F23.1 anti-T-cell receptor monoclonal antibody immobilized on Sepharose beads. We show that CR cross-linking of resting murine CD8$^+$ T cells seeded at low cell densities is insufficient to induce responsiveness to the growth-promoting effect of interleukin-2 (IL-2), i.e. fails to induce expression of functional IL-2 receptors. The macrophage cell-line product, IL-2 receptor-inducing factor (RIF), but not IL-1, IL-3, IL-4 and interferon-gamma (IFN-γ) functions efficiently as a co-stimulator. Once activated, growth of CD8$^+$ T cells is driven entirely by IL-2. We conclude that two restriction points control the activation of resting CD8$^+$ T cells. While cross-linking of TCR is essential as the first step, RIF is required as the competence factor to induce IL-2 responsiveness. We consider the possibility that the ability of APCs to produce RIF determines the immunogenicity of APCs towards antigen-reactive resting CD8$^+$ T cells.

INTRODUCTION

T cells have to be excited from their resting state to become susceptible to the action of proliferation-inducing factors such as IL-2 (Smith, 1980; Larsson, 1984; Nabholz & MacDonald, 1983). According to prevailing views, cross-linking of T-cell receptor structures (TCR) by specific or non-specific ligands induces in resting T cells the expression of functional (high-affine) IL-2 receptors, i.e. IL-2 responsiveness, while binding of autocrine or paracrine provided IL-2 promotes their growth and maturation (Wagner *et al.*, 1980; Erard *et al.*, 1985a; Erard, Nabholz & MacDonald, 1985b; Vohr & Hünig, 1985).

In analysing the minimal signal requirements for the induction of IL-2 responsiveness in resting murine CD8$^+$ T cells, we observed recently, in the Con A mitogen system, that in addition to cross-linking of cell-surface structures by the lectin, a co-stimulator activity is required (Hardt, Diamantstein & Wagner, 1985). This co-stimulator activity is provided either by accessory cells, or a soluble product thereof operationally termed IL-2 receptor-inducing factor (RIF) (Hardt *et al.*, 1985; Hardt, Sato & Wagner, 1987).

Originally we chose the Con A mitogen system because of the high frequency of responding cells (Pfizenmaier *et al.*, 1984).

Abbreviations: APC, antigen-presenting cells; CAS, conditioned medium (Con A); Con A, concanavalin A; HD, high density; IFN-γ interferon-gamma; N, lymphnode cells; mAb, monoclonal antibody; RIF, IL-2 receptor-inducing factor.

Correspondence: Dr H. Wagner, Dept. of Medical Microbiology and Immunology, University of Ulm, Oberer Eselsberg, 7900 Ulm, FRG.

The drawback of this model system is the interpretation of its physiological significance, since the target structures of Con A are ill defined. Presumably Con A is cross-linking multiple cell surface structures (Sitkowsky *et al.*, 1984). In order to study directly the effect of TCR cross-linking of resting CD8$^+$ T cells we resorted to the use of immobilized F23.1 anti-TCR antibodies (Staerz *et al.*, 1985), known to detect the products of limited numbers of $V_\beta 8$ genes (Sim & Augustin, 1985). Here we report that cross-linking of TCR is necessary but insufficient to induce IL-2 responsiveness in resting CD8$^+$ T cells. We show that RIF porovides the co-stimulator activity required.

MATERIALS AND METHODS

Mice

CBA (H-2^k), BALB/c and C57Bl/6 mice were bred in our own animal facilities with breeding pairs from Jackson Laboratories (Bar Harbor, ME), and were used at 4–6 weeks of age.

Preparation of high-density (HD) CD8$^+$ responder cells

As described elsewhere (Hardt *et al.*, 1987), mesenteric lymph node cells from 4–6-week-old mice were first passed over nylon-wool. The non-adherent cells were treated with a mixture of anti-I-A, anti-I-E and anti-L3T4 mAb plus complement. Thereafter, viable cells were purified and enriched for small, high-density (HD) cells by a discontinuous Percoll centrifugation step (Pharmacia, Uppsala, Sweden). The top fraction containing dead cells and the fraction containing medium-sized cells ($\delta = 1{\cdot}076$) were discarded, while the HD cells ($\delta = 1{\cdot}09$, usually 60–70% of the cell load) were recovered. Positive selection of

$CD8^+$ cells was performed by incubating 10^7 HD T cells with 150 μl fluorescein-conjugated anti-mouse $CD8^+$ mAb (Becton-Dickinson, Sunnyvale, CA) for 30 min on ice. Thereafter the cells were washed twice in cold serum-free phosphate-buffered saline (PBS), the stained cells sorted on an Epic V flow cytometer (Coulter Inc., Hialeah, FL), and the small $CD8^+$ cells collected on the basis of narrow-angle forward-light scatter and fluorescence intensity.

In some experiments, $F23.1^+$ T cells were selected from HD $CD8^+$ T cells by the planning method. HD $CD8^+$ T cells were labelled with F23.1 mAb (Staerz *et al.*, 1985) and were incubated twice in dishes coated with affinity-purified goat anti-rat Ig $F(ab)_2$ (Jackson Immuno-Research Laboratory Inc., Avondale, cat. No. 112 0572); after two consecutive absorption steps and washing off non-absorbing cells, adherent cells were recovered containing >90% $F23.1^+$ $CD8^+$ T cells.

Cytokine preparations

CAS (Con A supernatants) were produced as described elsewhere (Hardt *et al.*, 1985) from outbred rat spleen cells (5×10^6/ml) stimulated with 5 μg/ml Con A (Pharmacia) for 24 hr. RIF was purified partially from supernatants of the P388D1 macrophage cell line by batch elution from hydroxylapatite (Hardt *et al.*, 1987). Selected batches were purified further by subsequent fractionation using phenyl Sepharose, as described previously (Hardt *et al.*, 1987). Fractions were pooled that contained the main activity able to act as co-stimulator and referred to as IL-2 receptor-inducing factor (RIF). RIF bio-activity is associated with a 40,000 MW protein and separates from IL-1 (Hardt *et al.*, 1987). The RIF batches used here were devoid of IL-2 activity, and IL-4 activity was tested by [^{3}H]thymidine ([^{3}H]TdR) uptake by resting B cells stimulated with 5 μg/ml of goat anti-IgM.

Human recombinant IL-2 (rec.IL-2) purified from *E.coli* (98% IL-2 protein, <0·5 ng/ml LPS/mg protein, batch NP 50007 N09) was from Biogen, Geneva, Switzerland, and kindly donated by Dr H. J. Obert (Bioferon, Laupheim). Human rec. IL-1 and rec. β-IL-1 purified from *E.coli* and containing 10^8 thymocyte mitogenesis U/mg protein were kindly provided by St. Gillis (Immunex Corporation, Seattle, WA). Rec. IL-4 purified from yeast supernatants (Widmer & Grabstein, 1987), with an activity of 2×10^5 U/μg protein, was kindly donated by St. Gillis (Immunex Corporation). Rec. human TNF-alpha (batch 30), as well as monospecific rabbit anti-human TNF-alpha antiserum, was provided by Dr F. Frickel (Knoll AG, Ludwigshafen). Supernatants of the murine promyelomonocytic line WEHI-3 (subclone WEHI-3B (D-), a kind gift from Dr N. Iscove, Basel, Switzerland, were used as source rich in IL-3 bio-activity. Purified IL-3 was purchased from Genzyme (Boston, MA). Rat rec. IFN-γ containing 10^6 IU/ml was kindly provided by Dr H. Kirchner, DKFZ Heidelberg.

Monoclonal antibodies (mAb)

Anti-I-A^k (hybridoma 11-5-219) and anti-Thy-1.2 (hybridoma HO 13-4-9) were kindly provided by the Salk Institute, La Jolla, CA; anti-L3T4 (hybridoma Gk 1·5) was kindly provided by Dr Fitch, Chicago, IL; anti-I-E^k (hybridoma 17-3-35) and anti-IL-2R (hybridoma 7D4) were obtained from the American Tissue culture collection (Rockville, MD); anti-CD8 mAb (clone 53-6.7) came from Becton-Dickinson (cat. no. 1351), while the F23.1 hybridoma was kindly provided by Dr M. Bevan, La Jolla, CA, and anti-IL-4 mAb 11B11 was donated by Dr Paul (NIH, Bethesda, MD). Antibodies were purified from ascites fluid by passage over protein A–Sepharose CL4B (Pharmacia). Purified F23 mAb and anti-CD8 mAb were covalently coupled to CNBr-activated Sepharose 4B (Pharmacia). If not otherwise stated, 0·5 mg protein/ml Sepharose beads were coupled according to the Pharmacia Handbook for affinity chromatograpahy.

Accessory cells

Spleen cells from 4–6-week-old mice were freed of red cells with Tris-buffered NH_4Cl, and low-density (LD) cells ($\delta = 1{\cdot}062$) were recovered by a discontinous Percoll centrifugation. To deplete for T cells, LD cells were treated with anti-Thy-1.2 mAb plus complement.

Culture medium

The medium used was Click/RPMI-1640 (Biochrom, W. Berlin) supplemented with 10% (v/v) of a selected batch of fetal calf serum (Biochrom). FCS selection was critical; only FCS was used that gave little background proliferation of HD $CD8^+$ T cells co-cultured with F23.1 immobilized to Sepharose beads. Medium was supplemented with 10 mM HEPES buffer, 5×10^{-5} 2M-mercaptoethanol, 1 μg/ml indomethacin, 25 mM methyl-α-D-mannoside and antibiotics.

Culture conditions

If not stated otherwise, HD (resting) $CD8^+$ T cells from CBA mice were seeded at low cell densities (usually 1×10^3/culture) in U-shaped microwells (Greiner, Nürtingen) plus the reagents or cells listed. Stimulator cells (1×10^4 F23.1 hybridoma cells) were treated with 50 μg/ml mitomycin *c* (Sigma, Munich) at 37° for 20 min and were washed three times [^{3}H]TdR uptake was scored after pulsing each microculture with 0·6 μCi [^{3}H]TdR for 8 hr and harvesting the cells onto filters for liquid scintillation counting. The results are expressed as the mean of six replicate cultures. Standard deviation (SD) was less than 15%. For simplicity reasons SD values are omitted in the tables given.

RESULTS

Cross-linking of TCR is insufficient to induce IL-2 responsiveness

In order to investigate the effect of TCR cross-linking on resting $CD8^+$ T cells, high-density (resting) $CD8^+$ T cells were prepared from nylon-wool non-adherent murine LN cells according to the three-step procedure described previously (Hardt *et al.*, 1987). First, a negative selection step using a mixture of cytotoxic mAb (anti-L3T4, anti-I-A) plus complement was used to deplete for I-A^+ and CD4 cells. Second, by discontinuous Percoll density centrifugation high-density cells ($\delta = 1{\cdot}09$) were obtained. Third, viable high-density CD8 T cells were positively selected using an Epic cell sorter. Slightly less than 20% of the cells within highly purified resting $CD8^+$ T cells were stained by FITC-conjugated mAb F23.1 (data not given), indicating that the purification protocol did not significantly change the known ratio (16) of $F23^+/F23^-$ T cells within the $CD8^+$ T-cell subset.

When highly purified high-density (HD) $CD8^+$ T cells of CBA mice were cultured at low cell densities (10^3 cells/culture) together with mitomycin *c*-treated F23.1 hybridoma cells in the absence of exogenous IL-2, no proliferative response ensued (Table 1). However, in the presence of a surplus of exogenous

Table 1. Requirements for growth of resting Lyt-2+ T cells seeded at low cell density/culture

	Lyt-2+ T cells plus* rec. IL-2 (50 U/ml)	Addition of	Proliferative response (c.p.m.)
Experiment 1	+	—	300
	+	[F23.1 hybridoma]Mito	48,800
	+	[F23.1 hybridoma]Mito plus anti-IL-2R mAb†	4300
	(without rec. IL-2)	[F23.1 hybridoma]Mito	1200
Experiment 2			
	+	F23.1 Sepharose‡ plus CAS§	32,300
	+	F23.1 Sepharose	900
	+	F23.1 plus rec. IL-1 (20 U/ml)	1100
	+	F23.1 plus rec. IL-4 (2 ng/ml)	700
	+	F23.1 plus rec. TNF (1 μg/ml)	1400
	+	F23.1 plus IL-3 (10% v/v)	2300
	+	F23.1 plus IFN-γ (1 ng/ml)	700

* CBA mouse-derived HD Lyt-2+ T cells (1×10^3/well) were exposed to the various reagents or cells listed. Cell proliferation was measured at Day 4 by [^{3}H]TdR uptake. The data are given as mean c.p.m. of 6 microcultures. The SD of the mean was < 15%.

† Ascites diluted 1:50. Note that anti-L3T4 mAb did not inhibit cell proliferation (not shown).

‡ See the Materials and Methods.

§ Containing 8 U IL-2/ml.

IL-2 a striking proliferative response took place that was inhibitable by anti IL-2 receptor mAb (Table 1). Results of this type demonstrate that co-cultivation of limited numbers of CD8+ T cells with mitomycin-treated F23.1 hybridoma cells results in activation, i.e. induction of IL-2 responsiveness, presumably because membrane-bound anti-F23.1 antibodies cross-link the TCR structures of the responder cells. Assuming cross-linking of TCR alone but not additional ill-defined functions mediated by the hybridoma cells would convey IL-2 responsiveness, immobilized F23.1 mAb also ought to confer IL-2 responsiveness.

However, the additional results depicted in Table 1 indicate that this is not the case. While F23.1 mAb immobilized on Sepharose beads effectively triggered proliferative responses in the presence of crude Con A soup (CAS), known to contain a plethora of lymphokines besides IL-2, in the presence of rec. IL-2 alone no cell proliferation was observed. This type of result was obtained with CBA, C57Bl/6 and BALB/c mouse-derived resting CD8+ T cells. Furthermore, complementation of rec. IL-2 with rec. IL-1, rec. IL-4, rec. TNF, rec. IFN-γ and purified IL-3 was without effect. It should be noted that the lymphokines given were titrated over a large range of concentrations (data not given). We conclude that the stimulating properties of F23.1 hybridoma cells plus rec. IL-2 equals that of immobilized F23.1 mAb plus a mixture of lymphokines, as contained in CAS. Yet, F23.1 mAb immobilized on Sepharose plus rec. IL-2 alone lacks activation properties, even when mixed with defined lymphokines such as IL-1, IL-4, rec. TNF, IL-3 and IFN-γ. Obviously an additional bio-activity within CAS is critical for IL-2 responsiveness to become induced.

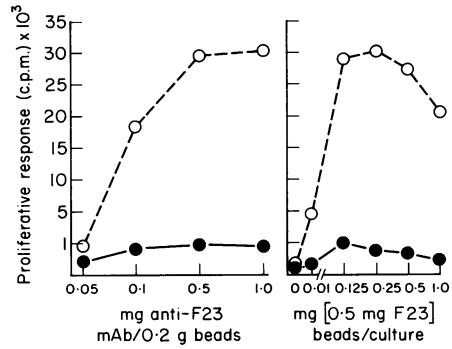

Figure 1. Activation of 2×10^3 HD Lyt-2+ T cells in the presence of 30 U/ml rec. IL-2 by F23 mAb immobilized on Sepharose beads alone (●) or by immobilized F23 mAb plus RIF (15% v/v) (○). (a) Shows various concentrations of F23.1 mAb used to couple Sepharose beads, (b) details mg beads used per culture, coupled with 0·5 mg F23 mAb.

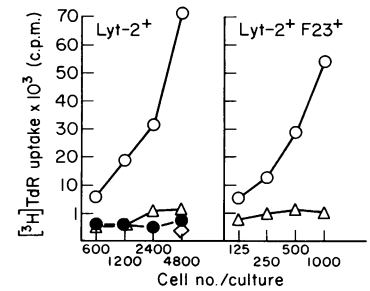

Figure 2. Activation of F23.1+ HD Lyt-2+. F23.1+ T cells were positively selected from HD Lyt-2+ T cells by panning the HD Lyt-2+ T cells on plastic coupled with F23.1 mAb. Proliferative responses obtained with graded cell concentrations/culture are compared. Immobilized F23.1 plus 50 U/ml rec. IL-2 (△), immobilized F23.1 mAb, 50 U/ml rec. IL-2 plus RIF (10% v/v) (○), immobilized Lyt-2+ mAb plus RIF plus rec. IL-2 (●), immobilized F23.1 plus RIF without IL-2 (◇).

RIF acts as co-stimulator

In mitogen (Con-A)-driven responses of resting CD8+ T cells plated at low cell densities, the induction of IL-2 responsiveness

Table 2. RIF controls IL-2 responsiveness

HD Lyt-2$^+$ T cells immobilized F23.1 mAb* plus 50 U/ml rec. IL-2	Addition of	Proliferative response (c.p.m.)
+	—	1200
+	Accessory cells (1×10^4/well)	57,000
+	RIF (25% v/v)	32,000
+	RIF (12·5% v/v)	27,000
+	RIF (6·25% v/v)	8000
+	RIF (1% v/v)	3200
(no rec. IL-2)	RIF (25% v/v)	700
(no immobilized F23.1 mAb)	RIF (25% v/v)	1300

* HD Lyt-2$^+$ T cells (1×10^3/well) were cultured over 5 days with 0·25 mg beads/culture coupled with 0·5 mg mAb. Semi-purified RIF was prepared and described in the Materials and Methods.

Table 3. Growth of resting HD Lyt-2$^+$ T cells* induced by immobilized F23.1 mAb plus RIF in response to IL-2 can be blocked by anti-IL-2R mAb but not by anti-IL-4 mAb

RIF (15% v/v)	IL-2 (20 U/ml)	Anti-IL-4 mAb (11B11)	Anti-IL-2R mAb (7D4) (final dilution)	Proliferative responses (c.p.m.)
+	+	−	1:50	1300
+	+	−	1:300	8400
+	+	−	1:2400	20,900
+	+	−	—	27,200
+	+	+	1:50	31,300
−	+			880

* HD Lyt-2$^+$ T cells (1×10^3/well) were cultured with 0·25 mg beads/culture coupled with 0·5 mg F23 mAb plus the various reagents listed. Cell proliferation was measured after 4 days by [^{3}H]TdR uptake.

is controlled by RIF, a lymphokine produced by the macrophage cell line P388D1 (Hardt *et al.*, 1985, 1987). Therefore, we analysed whether semi-purified RIF acts as co-stimulator for resting CD8$^+$ T cells exposed to F23.1 mAb immobilized on Sepharose beads. As shown in Fig. 1, mere cross-linking of TCR did not result in IL-2 responsiveness over a wide concentration of immobilized F23.1 mAb. However, in the presence of RIF a striking proliferative response was observed. Optimal proliferative responses were obtained using 0·2 mg beads/culture coupled with 0·5–1·0 mg F23.1 mAb (Fig. 1).

In order to exclude the possibility that immobilized F23.1 mAb failed to induce IL-2 responsiveness in resting CD8$^+$ T cells because of the numerical paucity of F23.1$^+$ cells within the responder cells, we also compared, over a wide cell concentration, the proliferative response of resting CD8$^+$ T cells to that of resting CD8$^+$ T cells positively selected for F23.1$^+$ responder T cells. As detailed in Fig. 2b TCR cross-linking of resting F23$^+$ CD8$^+$ T cells over a wide cell concentration again was insufficient to induce IL-2 responsiveness, i.e. growth in the presence of exogenous IL-2. Yet upon addition of RIF high proliferative responses occurred. Interestingly, when compared on a responder cell basis [^{3}H]TdR uptake is about five times higher using F23$^+$ CD8$^+$ T cells (Fig. 2a) compared with CD8$^+$ T cells (Fig. 2a), a factor that most likely is explained by the enrichment of F23$^+$ responder cells. Note that RIF functioned as co-stimulator only in combination with immobilized F23.1 mAb but not with immobilized anti-CD8 mAb (Fig 2a), a finding that stresses the special role of TCR cross-linking for the initiation of T-cell activation. That RIF acts not as progression factor is indicated by the fact that in the absence of exogenous IL-2 no proliferative response took place (Fig. 2a). We conclude from these data that TCR cross-linking is required but is insufficient to promote IL-2 responsiveness in resting CD8$^+$ T cells. To this, RIF is required as a co-stimulator and is able to substitute for accessory cells (Table 2). So far, however, our attempts have failed to recover significant RIF activity of culture supernatants of splenic-adherent cells or of F23.1 hybridoma cells (data not given).

Table 4. IL-2 alone, but not IL-1 sustains continuous proliferation of HD Lyt-2$^+$ upon activation with immobilized F23.1 mAb, RIF plus IL-2

Second-step culture (1×10^3 cells/well)	Addition of	Proliferative response (c.p.m.)
+	IL-2 (20 U/ml)	35,300
+	IL-2 plus anti-IL-2R mAb	9600
+	rec. IL-1 (20 U/ml)	1400
+	rec. IL-1 plus IL-2	34,200
+	—	900

* In a first-step culture HD Lyt-2$^+$ T cells (1×10^3/well) were activated by co-culture with immobilized F23.1 mAb, RIF and IL-2. After 3 days viable cells were harvested and co-cultured in a second-step culture with the reagents listed. Proliferative responses were recorded 3 days thereafter by asssaying [^{3}H] TdR uptake.

RIF acts as competence factor by inducing IL-2 responsiveness

Two types of experiments were performed to test whether RIF acts as competence factor. First, the inhibitory capacity of anti-IL-2 receptor mAb was examined. As shown in Table 3, anti-IL-2R mAb efficiently blocked the proliferative response of resting CD8$^+$ T cells towards immobilized F23.1 plus RIF and IL-2. This type of result strongly indicates that the proliferative response induced is exclusively triggered via the IL-2 receptor pathway, i.e. driven by IL-2.

Second, we tested whether upon activation of CD8$^+$ T cells by a combination of RIF plus IL-2 and immobilized F23.1, IL-2 alone would subsequently be sufficient to sustain proliferative responses. The results given in Table 4 demonstrate that only IL-2, but not for example rec. IL-1, supported the *in vitro* growth of short-term activated CD8$^+$ T cells. We conclude from these data that RIF acts as competence factor by inducing sensitivity to the growth-promoting effect of IL-2 in resting CD8$^+$ T cells excited by cross-linking of TCR structures.

DISCUSSION

We have analysed the signal requirements for the activation of HD (resting) murine CD8⁺ T cells by using the anti-T-cell receptor antibody F23.1 immobilized on Sepharose beads. Activation is defined here as those events that result in the expression of functional IL-2R, i.e. responsiveness to the growth-promoting effect of rec. IL-2. We describe that unlike F23.1 hybridoma cells, which are effective in inducing activation on resting CD8⁺ T cells, F23.1 mAb immobilized on Sepharose beads lack this ability. However, upon complementation of immobilized F23.1 mAb with semi-purified RIF, activation becomes efficiently induced. Furthermore, it is shown that RIF but not IL-1, IL-2 or IL-4, acts as competence factor by inducing IL-2 responsiveness in resting CD8⁺ T cells excited by TCR cross-linking. We conclude that in the model system studied, cell activation of resting CD8⁺ T cells represents a two-step event. Accordingly, cross-linking of TCRs induces sensitivity to the bio-activity of RIF, while RIF acts as competence factor for the induction of IL-2 responsiveness.

The RIF preparations used here were derived from culture supernatants of the macrophage cell line P388D1 (Hardt *et al.*, 1987). Semi-purified RIF is almost devoid of IL-1 activity and its bio-activity is associated with a 44,000 MW protein (Hardt *et al.*, 1987). In the lectin (Con A) system RIF acts as competence factor by inducing functional IL-2 receptors in resting CD8⁺ T cells exposed to Con A (Hardt *et al.*, 1985, 1987).

Interestingly, in the latter system Con A induces in HD CD8⁺ T cells detectable levels of c-myc mRNA, but not that of IL-2R mRNA. However, resting CD8⁺ T cells exposed to Con A plus RIF accumulate IL-2R mRNA within 18 hr, which then becomes further up-regulated by IL-2 (Hardt, 1987). Thus the molecular data available corroborate the functional data shown that RIF induces functional and phenotypical expression of IL-2R (Hardt *et al.*, 1985, 1987).

Others have shown that cross-linking of TCR induces rapid biochemical events, such as rise in $(Ca^{2+})i$ but that, unlike activated (cloned) T cells, a rise in $(Ca^{2+})i$ is not itself sufficient to induce IL-2R expression in resting T cells (Linch, Wallace & O'Flym, 1987). In humans, there is evidence that cross-linking of CD3 structures of resting T cells by anti-CD3 Sepharose beads is insufficient to activate resting T cells even in the presence of IL-1 and/or IL-2 (Linch *et al.*, 1987). While these data are in conflict with findings obtained in high cell density cultures (Palacios, 1985; Meuer & Meyer zum Büschenfelde, 1986), they clearly parallel the results described here. The conclusion that rapid and transient rise in $(Ca^{2+})i$ and early activation of protein kinase *C* that occurs on ligand–receptor interaction (O'Flym *et al.*, 1985) alone is inadequate to cause IL-2R expression in resting T cells points to a quantitative aspect. If it turns out that only *protracted* activation of protein kinase *C*, as for example obtained with phorbolesters (Weiss *et al.*, 1986), represents the biochemical signal required for IL-2R expression in resting T cells, then it could be that gross membrane perturbation alone can, under extreme circumstances, also lead to IL-2R expression. Indeed we repeatedly noted that absorption of F23.1 mAb onto *plastic beads*, which induces marked gross membrane perturbation of resting CD8⁺ T cells as evidenced by a unique stretched out morphological conformation, in part bypasses the co-stimulator requirement of RIF (data not given). This phenomenon, which again is dependent on the cell density of the CD8 responder cells plated, is analysed in more detail at present.

In this context recent results (Emmerich, Strittmatter & Eichmann, 1986; Eichmann *et al.*, 1987) obtained with human and mouse T lymphocytes need to be discussed. They point out that the fixation and immobilization of TCR relative to CD8 (CD8) or CD4 (L3T4) provides an optimal activation signal for T cells in the presence of exogenous factors contained within CAS. While confirming their data using CAS as source of IL-2, we recently noted that cross-linking of TCR and CD8 fails to induce responsiveness to rec. IL-2 alone. Thus concomitant cross-linking of TCR and CD8 structures does not circumvent the requirement for RIF (Schmidberger *et al.*, 1988). Related to the data described here is recent work by Staerz & Bevan (1986). Accordingly HD T cells plated at low cell densities to proliferate in response to immobilized F23.1 mAb plus CAS, yet fail to respond to immobilized F23.1 plus rec. IL-2. In the latter system the effect of RIF has not been evaluated.

The possibility that RIF bio-activity is related functionally and as a molecular entity to one of the defined lymphokines is of concern. However, neither rec. IL-1, rec. IFN-γ, rec. IL-4 and purified IL-3 can substitute for RIF under the experimental conditions used here. Clearly a final answer to this requires more extensive biochemical characterization of RIF. We also need to assay, as soon as it becomes available, in the model system used here, the effect of rec. IL-5 (Kinashi *et al.*, 1986) and rec. IL-6 (Yasukawa *et al.*, 1987).

Under physiological conditions, resting CD8⁺ T cells become activated by 'professional' antigen-presenting cells (APCs) such as dendritic cells (Steinman *et al.*, 1983). APCs are probably required at several levels, including presentation of antigen to cross-link TCR of antigen-reactive cells. The model system used here was designed to substitute APC-mediated cross-linking by an inert 'carrier' and subsequently to examine whether the further ligand signalling required for activation can be mediated by physiological lymphokines potentially produced by APCs. Unlike IL-1, IFN-γ, rec. TNF, IL-3 and rec. IL-4, RIF appears as promising candidate. Even though APCs are still twice as efficient compared with immobilized anti-F23.1 mAb plus RIF (Table 3), this view is strengthened further by our recent demonstration that in the presence of RIF and IL-2 resting CD8⁺ T cells mount both proliferative and antigen-specific cytolytic responses towards essentially non-immunogenic allogeneic class I MHC-expressing cells (to be published).

In conclusion, we show that TCR cross-linking by insolubilized F23.1 mAb is essential but insufficient to cause activation of resting CD8⁺ T cells. The missing second signal can be provided by RIF, which acts as competence factor in inducing IL-2 responsiveness. Whether RIF reflects the physiological 'co-stimulator' activity attributed to 'professional' APCs (Lafferty, Andrus & Prowse, 1980) requires further experimentation. The data available suggest that the ability of APCs to produce RIF represents a restriction point that determines the immunogenicity of antigen presented to antigen-reactive resting CD8⁺ T cells.

ACKNOWLEDGMENTS

The skilled technical assistance of Mrs R. Schmidberger is highly appreciated. We thank Dr P. Conradt for operating the cell sorter.

This work is supported by the SFB 322.

REFERENCES

EICHMANN K., JÖNSSON J.I., FALK I. & EMMERICH F. (1987) Effective activation of resting mouse T lymphocytes by crosslinking submitogenic concentrations of the T cell antigen receptor with either Lyt-2 or L3T4. *Eur. J. Immunol.* **17,** 643.

EMMERICH F., STRITTMATTER U. & EICHMANN K. (1986) Synergism in the activation of human CD8 T cells by crosslinking the T-cell receptor complex with the CD8 differentiation antigen. *Proc. natl. Acad. Sci. U.S.A.* **83,** 8298.

ERARD F., CORTHESY P., NABHOLZ M., LÖWENTHAL R., ZAECH P., PLAETNIK G. & MACDONALD H.R. (1985a) Interleukin 2 is both necessary and sufficient for the growth and differentiation of lectin-stimulated cytolytic T lymphocyte precursors. *J. Immunol.* **134,** 1644.

ERARD F., NABHOLZ M. & MACDONALD H.R. (1985b) Antigen stimulation of cytolytic T lymphocyte precursors: minimal requirements for growth and acquisition of cytolytic activity. *Eur. J. Immunol.* **15,** 798.

HARDT C. (1987) Activation of murine CD8 lymphocytes: two distinct signals regulate c-myc and interleukin-2 receptor RNA expression. *Eur. J. Immunol.* **17,** 1711.

HARDT C., DIAMANTSTEIN T. & WAGNER H. (1985) Signal requirements for the *in vitro* differentiation of cytotoxic T lymphocytes (CTL): distinct soluble mediators promote preactivation of CTL-precursors, clonal growth and differentiation into cytotoxic effector cells. *Eur. J. Immunol.* **15,** 472.

HARDT C., SATO N. & WAGNER H. (1987) Functional and biochemical characteristics of a murine interleukin 2 receptor-inducing factor. *Eur. J. Immunol.* **17,** 209.

KINASHI T., HARADA N., SEVERINSON E., TANABE T., SIDERAS P., KONISHI M. *et al.* (1986) Cloning of complementary DNA encoding T cell replacing factors and identity with B-cell growth factor II. *Nature (Lond.)*, **324,** 70.

LAFFERTY K.J., ANDRUS L. & PROWSE S.J. (1980) Role of lymphokine and antigen in the control of specific T cell responses. *Immunol. Rev.* **51,** 279.

LARSSON E.L. (1984) Activation and growth requirements for cytotoxic and noncytotoxic T lymphocytes. *Cell. Immunol.* **89,** 223.

LINCH D.C., WALLACE D.L. & O'FLYM K. (1987) Signal transduction in human T lymphocytes. *Immunol. Rev.* **95,** 137.

MEUER S.C. & MEYER ZUM BÜSCHENFELDE K.H. (1986) T cell receptor triggering induces responsiveness to interleukin 1 and interleukin 2 but does not lead to T cell proliferation. *J. Immunol.* **136,** 4106.

NABHOLZ M. & MACDONALD H.R. (1983) Cytolytic T lymphocytes. *Ann. Rev. Immunol.* **1,** 273.

O'FLYM K., ZANDERS E.D., LAMB J.R., BEVERLY P.C.L., WALLACE D.L., TATHAM P.E.R., TAX W.J. & LINCH D.C. (1985) Investigation of early T cell activation: analysis of the affect of specific antigen, interleukin 2 and monoclonal antibodies on intracellular free calcium concentration. *Eur. J. Immunol.* **15,** 7.

PALACIOS R. (1985) Mechanisms by which accessory cells contribute in growth of resting T lymphocytes initiated by OKT3 antibody. *Eur. J. Immunol.* **15,** 645.

PFIZENMAIER K., SCHEURICH P., DÄUBNER W., KRÖNKE M., RÖLLINGHOFF M. & WAGNER H. (1984) Quantitative representation of all T cells committed to develop into cytotoxic effector cells and/or interleukin 2 activity producing helper cells within murine T lymphocyte subsets. *Eur. J. Immunol.* **14,** 33.

SCHMIDBERGER R., MIETHKE T., HEEG K. & WAGNER H. (1988) Primary activation of murine CD8 T cells via crosslinking of T3 cell surface structures: two signals regulate induction of IL-2 responsiveness. *Eur. J. Immunol.* **18,** 277.

SIM G.K. & AUGUSTIN A.A. (1985) Vβ gene polymorphism and a major polyclonal T cell receptor idiotype. *Cell*, **42,** 89.

SITKOVSKY M., PASTERNAK M.S., LUGO J.P., KLEIN J.R. & EISEN H.N. (1984) Isolation and partial characterization of concanavalin A receptors on cloned cytotoxic T lymphocytes. *Proc. natl. Acad. Sci. U.S.A.* **81,** 1519.

SMITH K.A. (1980) T cell growth factor. *Immunol. Rev.* **51,** 336.

STAERZ U.D. & BEVAN M. (1986) Activation of resting T lymphocytes by a monoclonal antibody directed against an allotypic determinant on the T cell receptor. *Eur. J. Immunol.* **16,** 263.

STAERZ U.D., RAMMENSEE H.G., BENEDETTO J.D. & BEVAN M. (1985) Characterization of a murine monoclonal antibody specific for an allotypic determinant on a T cell antigen receptor. *J. Immunol.* **134,** 3994.

STEINMANN R.M., GUTSCHINOW B., WITMER M.D. & NUSSENZWEIG M.C. (1983) Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *J. exp. Med.* **157,** 613.

VOHR H.W. & HÜNIG T. (1985) Induction of proliferative and cytotoxic responses in resting Lyt2$^+$ T cells with lectin and recombinant interleukin 2. *Eur. J. Immunol.* **15,** 332.

WAGNER H., HARDT C., HEEG K., PFIZENMAIER K., SOLBACH W., BARTLETT R., STOCKINGER H. & RÖLLINGHOFF M. (1980) T–T cell interactions during cytotoxic T lymphocyte (CTL) responses: T cell derived helper factor (interleukin 2) as a probe to analyse CTL responsiveness and thymic maturation of CTL progenitors. *Immunol. Rev.* **51,** 215.

WEISS A., IMBODEN J., HARDY K., MANGER B., TERHORST C. & STOBO J. (1986) The role of the T3/antigen receptor complex in T-cell activation. *Ann. Rev. Immunol.* **4,** 593.

WIDMER M.B. & GRABSTEIN K.H. (1987) Regulation of cytolytic T lymphocyte generation by B-cell stimulatory factor. *Nature (Lond.)*, **326,** 795.

YASUKAWA K., HIRANO T., WATANABE Y., MURATANI K., MATSUDA T., NAKAI S. & KISHIMOTO T. (1987) Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6) gene. *EMBO*, **6,** 2939.