Brief Definitive Report

DIFFERENTIAL REQUIREMENTS FOR THE INDUCTION OF INTERLEUKIN 2 RESPONSIVENESS IN L3T4$^+$ AND Lyt-2$^+$ T CELL SUBSETS

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The induction of proliferation of resting T lymphocytes involves triggering by specific antigen or by mitogen as well as the release and consumption of the growth factor interleukin 2 (IL-2) (reviewed in reference 1). It has been difficult, however, to investigate the need for additional stimuli, such as cell-cell interactions, or for other soluble factors in these processes, because until recently studies could only be performed with heterogeneous populations of responding cells and crude sources of lymphokines. Thus, it is not known whether the two major subclasses of mature T lymphocytes, i.e., cytolytic T cells of the major histocompatibility complex (MHC) class I–restricted phenotype (Lyt-2$^+$ L3T4$^-$; designated Lyt-2$^+$) and the T helper cells of MHC class II–restricted phenotype (Lyt-2$^-$ L3T4$^+$; designated L3T4$^+$) require the same induction signals.

In an attempt to clarify this issue, we have recently demonstrated that highly purified Lyt-2$^+$ cells could be activated by lectin (2) or allogeneic cell lines of various origins (3) to proliferate in response to purified recombinant IL-2 in the absence of other cells or exogeneously supplied factors. We now show that purified L3T4$^+$ lymphocytes require an additional activation signal to become responsive to IL-2.

Materials and Methods

Mice. Adult female C57BL/6 mice were obtained from the Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland.

Reagents. Phorbol-12-myristate-13-acetate (PMA) was obtained from Sigma Chemical Co (St. Louis, MO). Ionomycin was obtained from Calbiochem-Behring Corp. (La Jolla, CA). Leukoagglutinin (LA) and concanavalin A (Con A) were obtained from Pharmacia (Uppsala, Sweden).

Culture Medium. Lymphocyte cultures were performed in Dulbecco's modified Eagle's medium supplemented as described (2).

Lymphokines. Recombinant IL-2 of human origin, purified to homogeneity according to polyacrylamide gel electrophoresis and free of endotoxin, was provided by Biogen S.A., Geneva, Switzerland. The biological activity corresponded to 0.3 ng protein per

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unit, where 1 unit produces 50% of the maximal proliferation of IL-2-dependent CTLL cells. Interleukine 1 (IL-1) was prepared from the supernatant of ultraviolet-irradiated P388D1 cells and was fractionated on phenyl Sepharose as described elsewhere (4).

Monoclonal Antibodies. Rat monoclonal antibodies (mAb) directed against nonpolymorphic determinants of L3T4 (H129.19 and RL172.4) and Lyt-2 (53-6.7 and 3.168) were kindly provided by Drs. M. Pierres, R. Ceredig, J. Ledbetter, and F. Fitch, respectively.

Cell Purification, Flow Microfluorometry, and Cell Sorting. Cortisone-resistant thymocytes (CRT) from C57BL/6 mice were obtained 48 h after a single intraperitoneal injection of 4 mg hydrocortisone acetate (Hydrocortifor; Vifor, Geneva). The cells (5×10^7/ml) were incubated for 45 min at 37°C with IgM mAb directed against either Lyt-2 (3.168) or L3T4 (RL172.4) and a 1:20 final dilution of rabbit complement (Cederlane Laboratories, Hornby, Ontario, Canada). The recovered cells were passed over Ficoll and washed twice with mixed leukocyte culture (MLC) medium.

Lyt-2- or L3T4-enriched cells (8×10^6/ml) were stained, respectively, with biotinylated anti-Lyt-2 (53-6.7) mAb followed by dichlorotriazinyl amino fluorescein–coupled avidin or with anti-L3T4 (H.29-19) mAb in combination with fluorescein isothiocyanate–coupled rabbit anti-rat Ig (Nordic Immunological Laboratories, Tilburgh, The Netherlands) and sorted sterilely on a FACS II flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA) as described previously (2). Small Lyt-2^+ or L3T4^+ lymphocytes were collected and the purity of the selected cells was determined by re-passing 2×10^4 positively selected cells.

Cell Cultures. Microcultures were established in round-bottomed microwells, with 1,000 or 2,000 purified L3T4^+ or Lyt-2^+ cells, in 200 μl MLC medium containing 200 U/ml recombinant IL-2 and various stimuli. Cultures were scored for cell proliferation on day 5 or 6. Microcultures inoculated with (on average) 25 cells or less were scored on day 9 for colonies of >50 large blast cells. Minimal estimates of the frequency of growth-inducible cells were made according to the method of Taswell (5).

Assay for Cell Proliferation. 1 μCi methyl-[^{3}H]thymidine (Amersham International Ltd., Amersham, United Kingdom) was added to 50 μl of cell suspension in a 6 h pulse. Standard deviations of [^{3}H]thymidine incorporation were determined from a minimum of four replicate microcultures.

Results and Discussion

To compare directly the activation requirements of L3T4^+ and Lyt-2^+ resting T cells, we used a microculture system in which small numbers of highly purified cells are cultured in the absence of accessory cells (2, 3). An example of the purification of L3T4^+ and Lyt-2^+ subsets is shown in Fig. 1. Beginning with CRT (a population containing predominantly either L3T4^+ or Lyt-2^+ cells), we routinely obtained purities of >99.5% by following a two-step procedure of negative selection (with IgM mAb plus complement) followed by positive selection on the FACS. These populations were then cultured at low cell density (5×10^3 to 10^4 per milliliter) with various combinations of stimuli including lectin (LA or Con A), phorbol ester (PMA), and calcium ionophore (ionomycin). Saturating concentrations of IL-2 (200 U/ml) were added to all microcultures to ensure optimal growth of appropriately activated (i.e., IL-2 responsive) cells.

Using this sensitive system, striking differences were observed in the activation requirements of L3T4^+ and Lyt-2^+ cells. Whereas Lyt-2^+ cells were induced to high levels of IL-2-dependent proliferation by PMA, ionomycin, or lectin, none of these stimuli resulted in significant proliferation by the L3T4^+ subset (Table I). This dissociation was not due to differences in the dose response of the two subsets, as shown by titrations over a wide range of PMA, ionomycin, or lectin concentrations (Fig. 2). These data confirm and extend earlier experiments in

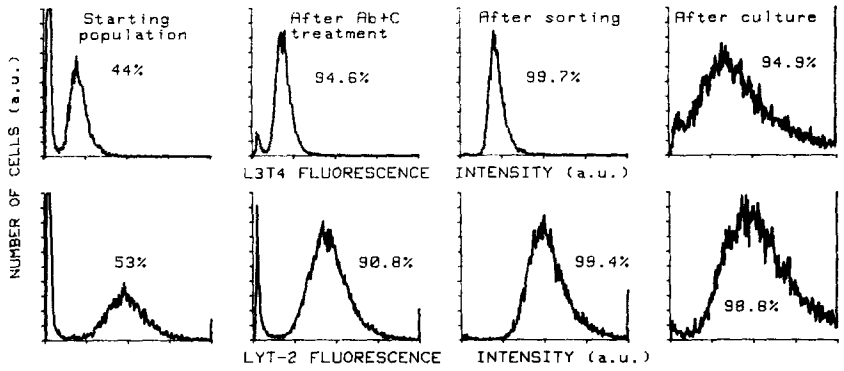

FIGURE 1. Purification of L3T4$^+$ and Lyt-2$^+$ subsets. CRT from C57BL/6 mice were first treated with IgM mAb against either Lyt-2 (3.168) or L3T4 (RL172.4) plus complement. The recovered cells (enriched in L3T4$^+$ or Lyt-2$^+$ cells, respectively) were then passed over Ficoll-Hypaque and stained with mAb against L3T4 (H129.19) or Lyt-2 (53.6.7). Positive cells were sorted (sterilely) on a FACS II. Purified Lyt-2$^+$ or L3T4$^+$ cells (1,000 per microwell) were then cultured for 6 d in the presence of PMA (1 ng/ml) plus IL-2 (200 U/ml) or PMA plus ionomycin (250 ng/ml) plus IL-2, respectively. The proportion of L3T4$^+$ (*top*) or Lyt-2$^+$ (*bottom*) cells at each stage of purification was determined by staining with mAb H129.19 or 53.6.7 as described in Materials and Methods.

TABLE I

Differential Requirements for the Induction of IL-2 Responsiveness in T Cell Subsets

Stimulus	Proliferation*	
	Lyt-2$^+$	L3T4$^+$
—	0.8 ± 0.4 (<1)	0.9 ± 0.2 (<1)
IL-2	1.4 ± 0.5 (<1)	0.9 ± 0.1 (<1)
PMA + IL-2	139 ± 11 (150)	0.7 ± 0.2 (<1)
LA + IL-2	127 ± 22 (156)	2.7 ± 2.7 (1)
Ionomycin + IL-2	51 ± 4 (28)	0.9 ± 0.1 (<1)
PMA + LA + IL-2	90 ± 6 (93)	15 ± 6 (7)
PMA + Ionomycin + IL-2	156 ± 15 (175)	87 ± 7 (54)
LA + Ionomycin + IL-2	84 ± 2 (38)	1.6 ± 1.4 (<1)
IL-1 + Ionomycin + IL-2	ND‡	0.7 ± 0.2 (<1)
IL-1 + LA + IL-2	ND	1.4 ± 0.3 (<1)

Purified Lyt-2$^+$ or L3T4$^+$ cells (1,000 per well) were cultured in 200 μl MLC medium containing various combinations of PMA (1 ng/ml), LA (2.5 μg/ml), ionomycin (250 ng/ml), IL-2 (200 U/ml), or IL-1 (1:50 dilution of a concentrated P388D$_1$ supernatant), as indicated. On day 6, viable cells were counted and [^{3}H]thymidine incorporation was measured.

* Results are expressed as mean dpm ($\times 10^{-3}$) ± 1 SD of quadruplicate microcultures. Values in parentheses represent viable cells ($\times 10^{-4}$).

‡ Not done.

which Con A was shown to preferentially induce the IL-2-dependent proliferation of the Lyt-2$^+$ subset in both bulk (6) and limiting dilution (7) cultures, as well as more recent studies of Malek et al. (8) indicating that semipurified Lyt-2$^+$ (but not L3T4$^+$) cells express high levels of IL-2 receptors after Con A stimulation.

Since none of the stimuli tested could by themselves induce IL-2 responsiveness in L3T4$^+$ T cells, we looked for synergistic effects. As shown in Table I and Fig. 2, the combination of PMA plus ionomycin produced maximal proliferation of purified L3T4$^+$ T cells. In fact, under these culture conditions, L3T4$^+$ cells increased ~100-fold in absolute cell number to reach one-third the maximal cell

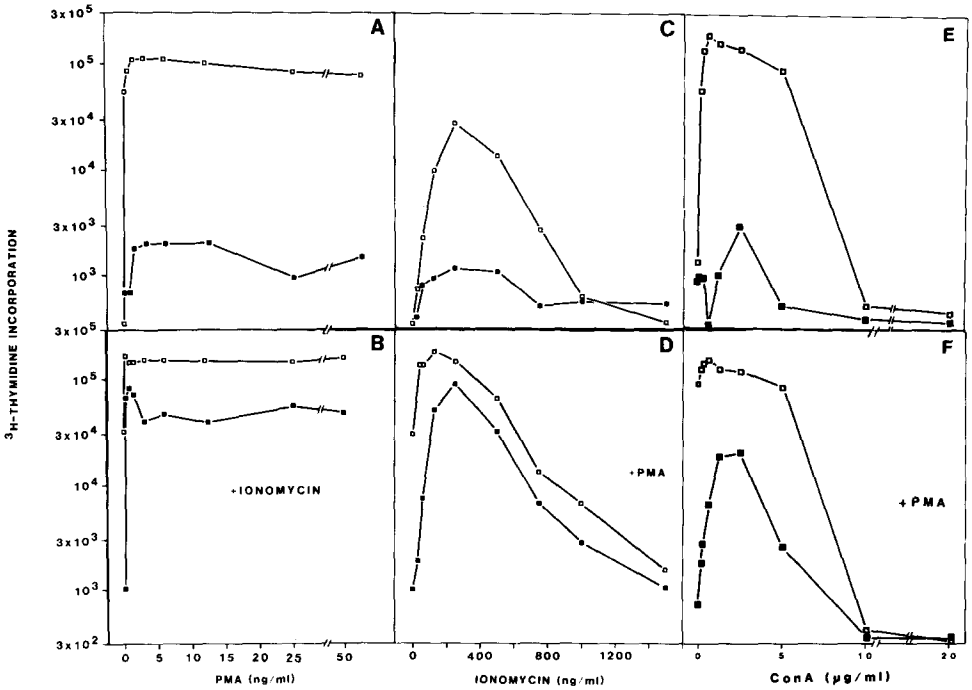

FIGURE 2. Dose response analysis of the stimulation of purified Lyt-2$^+$ or L3T4$^+$ cells by combinations of PMA, lectin, or calcium ionophore. Purified Lyt-2$^+$ (□) or L3T4$^+$ (■) cells (1,000 per well) were cultured in 200 μl MLC medium with the indicated concentrations of PMA (*A*, *B*), ionomycin (*C*, *D*) or Con A (*E*, *F*). Recombinant IL-2 (200 U/ml) was added to these microcultures either alone (*A*, *C*, *E*) or together with constant (optimal) amounts of ionomycin (250 ng/ml; *B*) or PMA (1 ng/ml; *D* and *F*). On day 6, [^{3}H]thymidine incorporation was measured on a fraction (50 μl) of each microculture.

density of Lyt-2$^+$ cells (Table I). Growth was not due to rare contaminants since >95% of cultured cells expressed the L3T4 phenotype after 6 d (Fig. 1). Optimal doses for this synergistic effect were 250 ng/ml for ionomycin and >1 ng/ml PMA (Fig. 2). Synergistic effects (10–20-fold) on the IL-2-dependent proliferation of L3T4$^+$ cells were also observed with combinations of PMA and lectin (either LA or Con A); however, no synergism was observed between lectin and ionomycin over a wide range of concentrations (Table I and unpublished data).

The synergistic effects of PMA plus lectin or PMA plus ionophore on the IL-2-dependent proliferation of L3T4$^+$ cells are consistent with a number of recent studies suggesting a two signal model for T helper cell activation. Thus, IL-2 secretion by the human T cell leukemia line Jurkat can be induced by combinations of PMA plus phytohemagglutinin or PMA plus ionomycin (9). Moreover, PMA plus ionomycin (10) or PMA plus Con A (8) have been shown to stimulate proliferation and/or IL-2 receptor expression by murine T lymphocytes or semipurified L3T4$^+$ cells, respectively. Together with the present results, these data suggest that "activation" of L3T4$^+$ cells requires at least two signals, one of which can be provided by PMA and the other by lectin or ionophore. In contrast, Lyt-2$^+$ cells can be activated by either signal; however, we cannot formally exclude the possibility that distinct subsets of Lyt-2$^+$ cells respond to PMA and ionomycin, respectively.

It has been suggested (11) that the macrophage product IL-1 is involved in the activation of $L3T4^+$ cells and that this requirement can be replaced by PMA. In our system IL-1 did not substitute for PMA in the synergistic stimulation of purified $L3T4^+$ cells by either LA or ionomycin (Table I). These data are consistent with another recent report (8) in which PMA (but not IL-1) was able to synergize with Con A in the induction of IL-2 receptor expression on $L3T4^+$ cells.

The question of whether accessory cells and/or cell-to-cell contact are required for T lymphocyte activation remains controversial. In this context, we recently demonstrated (2) that a single $Lyt\text{-}2^+$ lymphocyte could be induced to proliferate in the presence of LA plus IL-2. We now report a similar result for $L3T4^+$ cells stimulated by a combination of PMA, ionomycin, and IL-2. Under these conditions, the frequency of growing $L3T4^+$ cells in one experiment was almost as high as that of $Lyt\text{-}2^+$ cells (42 vs. 63%). In a second experiment, with lower plating efficiency, 14% of micromanipulated $L3T4^+$ cells grew, as compared with the 9% predicted by limiting dilution analysis of the same population. Thus neither accessory cells nor cell-to-cell contact is obligatory for the growth of $L3T4^+$ cells.

In conclusion, our data suggest that the intracellular pathways leading to T lymphocyte "activation" (as measured by IL-2 responsiveness) are more complex in the $L3T4^+$ subset. The requirement for specialized Ia^+ accessory cells in the alloantigenic stimulation of $L3T4^+$ (but not $Lyt\text{-}2^+$) T lymphocytes (12) may be a physiological reflection of this complexity.

Summary

Minimal requirements for the induction of interleukin 2 (IL-2) responsiveness in purified subsets of murine T lymphocytes have been investigated. Whereas $Lyt\text{-}2^+$ cells could be induced to IL-2-dependent growth by lectin, phorbol ester, or calcium ionophore, none of these stimuli was by itself sufficient for $L3T4^+$ cells. The latter cells could, however, be induced to respond to IL-2 by combinations of lectin plus phorbol ester or ionophore plus phorbol ester (but not lectin plus ionophore). Under optimal conditions, growth of $L3T4^+$ cells (like $Lyt\text{-}2^+$ cells) was independent of accessory cells and cell-cell contact.

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