Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 6268–6271, September 1985
Immunology

Interleukin 2 promotes growth and cytolytic activity in human $T3^+4^-8^-$ thymocytes

(T-lymphocyte differentiation/T-cell precursor/T3–receptor complex/cytotoxic thymocytes/interleukin-2 receptor)

ANTONIO DE LA HERA, MARIA LUISA TORIBIO, CARLOS MARQUEZ, AND CARLOS MARTINEZ-A.

Department of Immunology, Clinica Puerta de Hierro, Universidad Autonoma, San Martin de Porres 4, 28035 Madrid, Spain

Communicated by Frank Lilly, May 20, 1985

ABSTRACT **Human thymocytes bearing T3 but neither T4 nor T8 antigens ($T3^+4^-8^-$ cells) were obtained after negative selection of thymocytes, either fresh or cultured in medium containing recombinant interleukin 2 (IL-2), by treatment with Na1/34, OKT4A and B9.4 monoclonal antibodies (which recognize T6, T4, and T8 antigens, respectively) and complement. Quantitative flow cytometry showed a 98% pure population of $T3^+4^-8^-$ lymphocytes, which included proliferating cells. The growth and maturation requirements of these thymocytes were characterized and related to the T3–receptor complex and IL-2 pathways, thought to be used by mature lymphocytes. The results show that addition of recombinant IL-2 promotes, in a dose-dependent way, proliferation and acquisition of effector functions by cultured $T3^+4^-8^-$ thymocytes, the growth being inhibitable by monoclonal antibody 33B73 (anti-Tac). Furthermore, cytolytic activity of $T3^+4^-8^-$ cells induced by recombinant IL-2 is specifically blocked by monoclonal antibody OKT3, showing that it operates via the T3–receptor complex and does not require either T4 or T8 molecules. The finding of *in vitro* responsiveness to recombinant IL-2 in $T3^+4^-8^-$ thymocytes suggests a role of IL-2 in the growth and maturation of cells committed to the T-cell lineage, during intrathymic differentiation, prior to expression of T4 and T8 molecules.**

Cytotoxic T-lymphocyte (CTL) function is acquired, after initial recognition, in a two-step process involving growth and differentiation of CTL precursors (CTL-p) (1). Triggering mediated by antigen–receptor complex results in both interleukin 2 (IL-2)-receptor expression and clonal expansion through an IL-2-dependent autocrine pathway (2). Induction of IL-2-receptor expression by antigen, monoclonal antibodies (mAbs), or lectins is transient (3), allowing only limited clonal amplification. Furthermore, IL-2 is able to regulate expression of its own receptor (4, 5). Thus, the analysis of surface receptors implicated in recognition is of major interest in the understanding of T-lymphocyte activation, for initial triggering, entrance into the IL-2 pathway, or development of effector function (6). Present models for CTL recognition (7, 8) propose the implication of a minimal array of critical glycoproteins: the T3–receptor complex (9) and the subset-restricted T4/T8 companions (10). This phenotype combination, which is acquired at a discrete stage of intrathymic differentiation (11, 12), defines mature T cells (13) and includes all CTL-p and CTL (6). We have now assessed the role of these critical glycoproteins (T3, T4, and T8), using both thymic subpopulations and recombinant IL-2 (rIL-2), by phenotypic and functional analysis. We show the existence of CTL-p using the IL-2 pathway, within a proliferating $T3^+4^-8^-$ thymocyte population, where IL-2 is a sufficient *in vitro* requirement to promote proliferation and acquisition of cytolytic activity.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Isolation of Lymphocyte Populations. Single-cell suspensions were obtained from normal thymus fragments that had been removed during corrective cardiac surgery of patients 2 months to 5 years old. Viable thymocytes and peripheral blood mononuclear cells (PBL) were isolated by Ficoll-Hypaque density centrifugation. Thymocytes were immunoselected with the indicated mAb plus complement, as described (14). Briefly, either fresh or cultured cells were incubated with saturating amounts of mAb(s) for 30 min at 4°C, followed by 45 min at 37°C with a 1:5 dilution of noncytotoxic rabbit complement (Beringwerke, Marburg, F.R.G.).

mAbs. OKT3, OKT4A, OKT8, and OKT11 were purchased from Ortho Diagnostics. CD1 (Thy, gp45,12), referred to as Na1/34 (15), and CD8 (T, gp32,33), referred to as B9.4 (16), recognize T6 and T8 antigen, respectively (17). mAb 33B73 is specific for the 55-kDa IL-2 receptor structure, expressed as a T-cell activation antigen (Tac) (18). W6/32 specificity is a nonpolymorphic determinant on HLA A, B, and C antigens (19). B9.4 and 33B73 were provided by C. Mawas and D. Olive (Centre d'Immunologie, Marseille-Luminy, France).

Quantitative Flow Cytometry. The procedure for indirect immunofluorescence staining of the cells has been described (14). Fluorescein-conjugated $F(ab'_2)$ goat anti-mouse IgG (Kallestad Laboratories, Austin, TX) or goat anti-rat IgG (Nordic, Tilburg, Netherlands) were used as second-step reagents. Quantitation of the surface staining of 10^4 viable cells was performed with an EPICS-C flow cytometer (Coulter). Cells that stained above the second-step reagent background were considered positive.

Proliferation Assays. Cultures (0.2 ml) were set up in RPMI 1640 medium supplemented with 2 mM glutamine, 10 mM Hepes, and 10% (vol/vol) human serum (complement-depleted and pooled type AB). $T3^+4^-8^-$ thymocytes (1.5×10^5) were cultured in medium either with or without rIL-2 (Hoffmann–LaRoche, Basel, Switzerland); inhibition of proliferation was tested in the presence of rIL-2 at 12 units/ml and different dilutions of mAb 33B73. Cells were incubated for 5 days, with [*methyl*-H^3]thymidine (Radiochemical Centre) (1 μCi per 0.2-ml culture; 1 Ci = 37 GBq) present for the last 8 hr, and harvested afterwards onto glass-fiber filters. The amount of radiolabel incorporated was determined by liquid scintillation spectroscopy. Results are expressed as mean cpm of [*methyl*-H^3]thymidine incorporated, in triplicate cultures, and the percent inhibition of its incorporation at each 33B73 dilution.

Abbreviations: CTL, cytotoxic T lymphocyte(s); CTL-p, CTL precursor(s); IL-2, interleukin 2; rIL-2, recombinant IL-2; mAb, monoclonal antibody; PBL, peripheral blood lymphocyte(s).

CTL Assays. PBL or thymocytes, cultured in 2-ml macrowell plates (3×10^6 cells per well) with rIL-2 at 25 units/ml, were tested for cytotoxicity against 5×10^3 ^{51}Cr-labeled K562 cells at various effector-to-target ratios in a 4-hr ^{51}Cr-release assay. Results show the mean specific ^{51}Cr-release (%) in triplicate wells (14). Selective inhibition of the cytolytic activity was tested in either the absence or the presence of OKT3, OKT4A, OKT8, or W6/32, as described (14, 20). Indirect immunofluorescence with mAb W6/32 shows that all effector cells express HLA class I antigen (data not shown).

RESULTS

Presence of $T3^+4^-8^-$ Lymphocytes Within Thymus. We have consistently observed induction of proliferation and generation of CTL when $T3^+$ thymocytes were cultured with either rIL-2 or semipurified IL-2-containing culture supernatants (14). To characterize the cells responsible for this phenomenon, we performed quantitative flow-cytometric analysis with the T-cell differentiation-cluster mAbs (Table 1). As previously described (13, 21), the thymus comprises a heterogeneous population of $T11^+$ lymphocytes, of which about 30% are mature $T3^+$ thymocytes and about 70% are immature $T6^+$ cells. Depletion with Nal/34 plus complement leaves an enriched population of $T3^+$ cells (80–90%) where, surprisingly, $T4^+$ plus $T8^+$ lymphocytes do not account for the whole $T3^+$ subset, suggesting the existence of $T3^+4^-8^-$ thymocytes. Further immunoselection, using OKT4A and B9.4 plus complement, directly shows the existence of a minor (0.5–3%) thymocyte population of $T3^+4^-8^-$ phenotype, not previously described (13, 21). In contrast, a phenotypic profile of PBL, using the same procedure shown in Table 1 and elsewhere (13), reveals that $T4^+$ plus $T8^+$ cells account for the whole population of $T3^+$ lymphocytes in peripheral blood.

When thymocytes depleted of $T6^+$ cells are cultured with IL-2, the cells recovered, which include both mature thymocytes ($T3^+4^+8^-$ and $T3^+4^-8^+$) and $T3^+4^-8^-$ cells, grow for up to 10–12 days but not for longer periods, implying the absence of ubiquitous triggering signals in our culture conditions. This growth parallels the maintenance of the phenotypic profile of these cultures, where $T3^+4^-8^-$ cells constitute 25–35% of the whole. Furthermore, after treatment with OKT4A, B9.4, and complement, a >98% pure population of $T3^+4^-8^-$ cells was obtained, as judged by flow cytometry as described above for freshly isolated thymocytes (data not shown). It is of note that (*i*) a variable percentage (up to 15%) of these cells constitutively express IL-2 receptors, in contrast to the absence of expression in PBL, and (*ii*) 14% and 9% of these cells are found in S and G_2+M phases of the cell cycle, respectively, as assessed by quantitation of DNA content after propidium iodide staining (22), whereas this feature is absent in PBL (data not shown). These observations indicate that the $T3^+4^-8^-$ population represent *in vivo* proliferating thymocytes (22).

Table 1. Quantitative flow-cytometric analysis of human thymocytes and PBL

Cell type	Treatment*	% of cells positive					
		T6	T11	T3	T4	T8	Tac
Thymocytes	None	72	97	31	65	51	<1
	Nal/34 + C′	<1	ND	88	32	9	2
	Nal/34 + C′ and (OKT4 + B9.4) + C′	<1	93	81	<1	<1	16
PBL	None	<1	73	76	51	19	<1

mAbs used were Nal/34 (T6), OKT11 (T11), OKT3 (T3), OKT4A (T4), B9.4 (T8), and 33B73 (Tac). Results are representative of experiments with 10 thymus samples. ND, not done.
*See *Materials and Methods*; C′, complement.

Characterization of Growth and Effector-Function Requirements of $T3^+4^-8^-$ Thymocytes. To analyze the requirements for the growth and induction into effector function of the $T3^+4^-8^-$ thymocytes, we cultured them in medium containing rIL-2. As shown in Fig. 1, the presence of rIL-2 increases thymidine incorporation in a dose–dependent way. Furthermore, inhibition of rIL-2-promoted thymidine incorporation correlates with the dilution of anti-Tac mAb added to the culture (Fig. 2), showing that IL-2 is acting via its own receptor.

Functional analyses were carried out with $T3^+4^-8^-$ thymocytes. Results in Fig. 3 illustrate the lack of cytotoxic activity both at the initiation (day 0) or after 7 days of culture in medium without IL-2. In contrast, when rIL-2 is present, cytotoxic activity is induced, implying that $T3^+4^-8^-$ are CTL-p thymocytes and only require the addition of IL-2 to the cultures to become fully competent. The molecules implicated in this cytolytic activity were analyzed by performing functional blockade with mAbs. As shown in Fig. 4, mAb OKT3 specifically blocks cytotoxicity in a dose-dependent way. These results demonstrate that this cytolytic activity is mediated by the T3–receptor complex, which is a feature classically attributed to CTL but absent in most natural killer cells from PBL (20, 23). Fig. 4 also shows that the cytotoxic activity of mature thymocytes grown in rIL-2 is inhibited by both OKT4 and OKT8, again a feature consistent with CTL activity (20, 24, 25), and absent in those cloned natural killer cell lines that use the T3–Ti complex (26). Furthermore, cytolysis and its inhibition by mAb OKT3 were observed when Daudi and Raji cells, which are resistant to natural killer cells, were used as targets in the cytotoxicity assays (data not shown). OKT4 and OKT8 do not inhibit the cytotoxic activity mediated by $T3^+4^-8^-$ cells, confirming the absence of contaminating $T4^+$ or $T8^+$ cells.

DISCUSSION

Here we report the existence of $T3^+4^-8^-$ thymocytes that constitutively express IL-2 receptor and that grow *in vitro* in medium containing rIL-2 in the absence of triggering signals. The presence of $T3^+4^-8^-$ thymocytes indicates an intrathy-

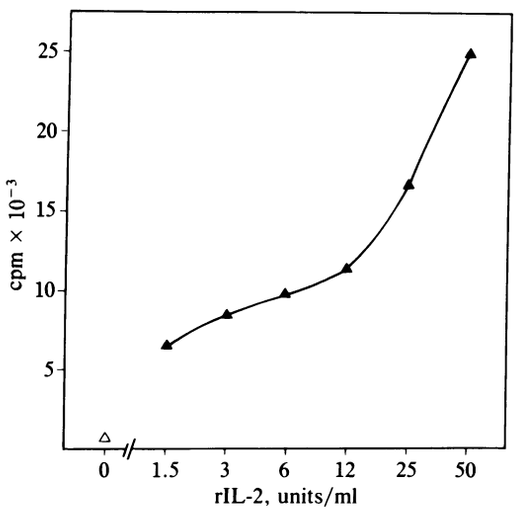

FIG. 1. rIL-2 promotes proliferation of $T3^+4^-8^-$ thymocytes in a dose-dependent way. $T3^+4^-8^-$ thymocytes were cultured either without (△) or with various amounts of rIL-2 (▲) for 5 days. Data represent the mean cpm of [*methyl*-H^3]thymidine incorporated in triplicate cultures.

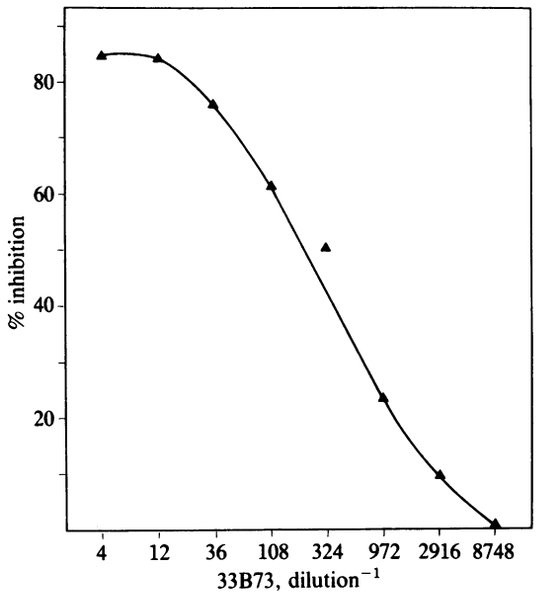

FIG. 2. Concentration of 33B73 correlates with the blockade of IL-2-mediated proliferation. $T3^+4^-8^-$ thymocytes were cultured with the indicated dilutions of hybridoma 33B73 culture supernatant, in the presence of rIL-2 at 12 units/ml. Data represent inhibition of the [^{3}H]thymidine incorporation observed in the absence of mAb (see Fig. 1). A 1:4 dilution of 33B73 supernatant did not affect thymidine incorporation by human T-cell leukemia line CEM (data not shown).

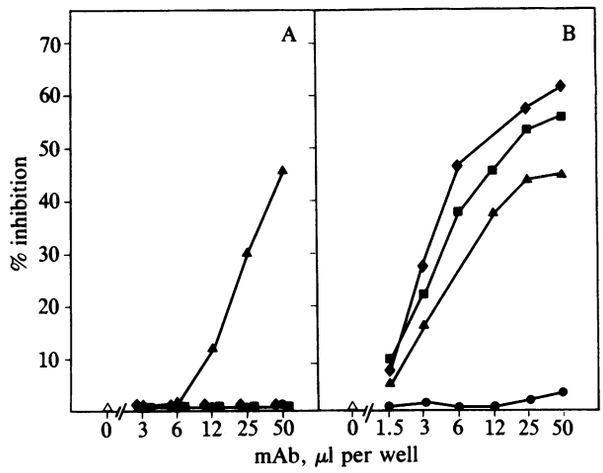

FIG. 4. Selective blockade of the cytotoxicity of cultured $T3^+4^-8^-$ cells by mAb OKT3. T6-depleted thymocytes were cultured for 7 days in medium containing rIL-2 at 25 units/ml. Recovered cells were either treated with OKT4A plus B9.4 plus complement to obtain $T3^+4^-8^-$ cells (*A*) or left untreated (*B*). Cytolytic activity of the cells was tested, as described in the legend for Fig. 3, in the absence (△) or presence of OKT3 (▲), OKT4A (◆), OKT8 (■), or W6/32 (●), as described (16, 17). The inhibition of cytolytic activity in the presence of the various amounts of mAb is shown. The specific ^{51}Cr release was 41% and 50% for $T3^+4^-8^-$ and $T6^-$ cultured thymocytes, respectively, at 25:1 effector/target ratio. Spontaneous release was <10%.

mic differentiation stage in addition to pre-T cells and mature thymocytes, out of stage II "common thymocyte" (13). Recently, ontogenic analyses in mice by Snodgrass *et al.* (27) have shown $MT4^-$ $Lyt\text{-}2^-$ 17-day fetal thymocytes already bearing the heterodimeric receptor. On the other hand, IL-2-receptor expression was observed in >50% of fetal mouse thymocytes at day 15 of gestation (28, 29), when thymocytes are $MT4^-$ $Lyt\text{-}2^-$ (30, 31). Taken together, these results show that the clonotypic receptor, T3 antigen, and IL-2 receptor are expressed early in ontogeny, prior to division into mature subpopulations; this conclusion is relevant for the understanding of the role of IL-2, T4, and T8 molecules in the acquisition and selection of T-cell repertoires. In this regard, it has been shown that mouse "double-negative" thymocytes fully regenerate all T-cell subpopulations *in vivo* (32), and we have observed mature-subset ($T3^+4^+8^-$ and $T3^+4^-8^+$) differentiation from human $T3^+4^-8^-$ precursors *in vitro* (unpublished work). These observations provide evidence that these $T3^+4^-8^-$ cells do not represent a dead-end intrathymic differentiation stage or a separate T-cell lineage. Furthermore, the existence of $T3^+4^-8^-$ thymocytes allows alternative explanations to the apparent discrepancy between antigen-receptor expression in mouse immature thymocytes (27) and its correlation to the presence of T3 in mature human thymocytes (11, 12).

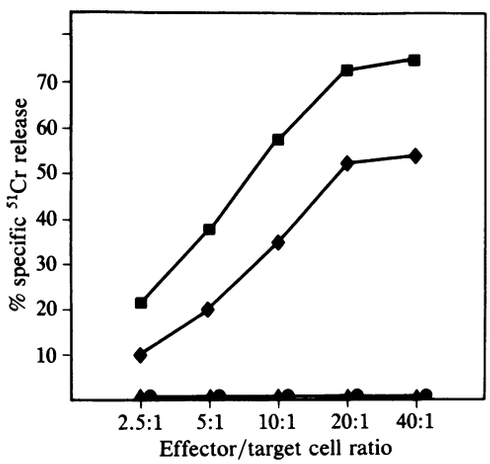

FIG. 3. Cytotoxic activity of human $T3^+4^-8^-$ thymocytes. $T3^+4^-8^-$ thymocytes, either freshly isolated (▲) or after culture for 7 days in medium with (◆) or without (●) rIL-2 were tested for cytotoxicity, in a 4-hr ^{51}Cr-release assay using ^{51}Cr-labeled K562 cells as targets. Percent specific release at various effector/target ratios is shown. As a control, the cytotoxic activity of human PBL (■) was assayed.

Our results also show that the addition of IL-2 alone also promotes maturation of $T3^+4^-8^-$ thymocytes into cytolytic effectors *in vitro*. Importantly, the origin of the cells constitutively responding to IL-2 can be ascribed to T-cell lineage rather than to others (i.e., natural killer cells) for the following reasons. (*i*) The $T3^+4^-8^-$ cells do not exhibit spontaneous (natural) cytotoxicity. (*ii*) They have the ability to kill natural killer-resistant target cells. (*iii*) Their cytotoxic activity is blocked specifically by mAb OKT3; such inhibition is an essential characteristic of CTL but not of most natural killer cells from PBL (6, 20, 23). (*iv*) They are $T3^+$ but lack the expression of either Leu-7, Leu-11, or M-1 antigens (data not shown and ref. 14). Thus, in agreement with previous reports on mouse peripheral cells (33, 34), T4/T8 molecules are not an absolute requirement for T-lymphocyte function.

The thymus plays a central role in T-lymphocyte differentiation and selection of repertoires. The physiological existence of functional $T3^+4^-8^-$ cells raises the question of the significance of their presence in the thymus, as well as the possibility of their migration into the periphery. On the other hand, Sakaguchi *et al.* (35) have recently described $Thy\text{-}1^+$ Lyt^- cells in normal mouse spleen. Furthermore the involvement of cells with equivalent phenotypes in autoimmune and lymphoproliferative disorders (refs. 35–37 and our unpublished observations) poses the requirement for regulatory

mechanisms operating in the periphery, after intrathymic selection.

We are grateful to Drs. C. Mawas and D. Olive (Centre d'Immunologie de Marseille-Luminy) for the generous gift of mAbs B9.4 and 33B73, to the members of the Pediatric Cardiosurgery Unit at C. E. Ramon y Cajal (Madrid) who provided the thymus samples, to Drs. A. Coutinho and R. R. Bernabe for reading the manuscript, to Mr. C. Aranda for skillful assistance in the immunofluorescence analysis, and to Coulter Cientifica for the facilities. The rIL-2 was kindly provided by Hoffmann-LaRoche (Basel). This work was partially supported by Comision Asesora de Investigacion Cientifica y Tecnica (CAICYT) Grant 1687/82 and by Fondo de Investigaciones Sanitarias de la Seguridad Social (FISS).

1. Larsson, E. L., Coutinho, A. & Martinez-A., C. (1980) *Immunol. Rev.* **51,** 61–91.
2. Meuer, S. C., Hussey, R. E., Cantrell, D. A., Hodgdon, J. C., Schlossman, S. F., Smith, K. A. & Reinherz, E. L. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1509–1513.
3. Cantrell, D. A. & Smith, K. A. (1983) *J. Exp. Med.* **158,** 1895–1911.
4. Welte, K., Andreeff, M., Platzer, E., Holloway, K., Rubin, B. Y., Moore, M. A. S. & Mertelsmann, R. (1983) *J. Exp. Med.* **160,** 1390–1403.
5. Reem, G. & Yeh, N. H. (1984) *Science* **225,** 429–430.
6. Reinherz, E. L., Acuto, O., Fabbi, M., Benussan, A., Milanese, C., Royer, H. D., Meuer, S. C. & Schlossman, S. F. (1984) *Immunol. Rev.* **81,** 95–144.
7. Reinherz, E. L., Meuer, S. C. & Schlossman, S. F. (1983) *Immunol. Today* **4,** 5–8.
8. Swain, S. L., Dutton, R. W., Schwab, R. & Yamamoto, J. (1983) *J. Exp. Med.* **157,** 720–729.
9. Meuer, S. C., Acuto, O., Hussey, R. E., Hodgdon, J. C., Fitzgerald, K. A., Schlossman, S. F. & Reinherz, E. L. (1983) *Nature (London)* **303,** 808–810.
10. Meuer, S. C., Hussey, R. E., Hodgdon, J. C., Hercend, T., Schlossman, S. F. & Reinherz, E. L. (1982) *Science* **218,** 471–473.
11. Acuto, O., Hussey, R. E., Fitzgerald, K. A., Protentis, S. P., Meuer, S. C., Schlossman, S. F. & Reinherz, E. L. (1983) *Cell* **34,** 717–726.
12. Royer, H. D., Acuto, O., Fabbi, M., Tizard, M., Ramachandran, K., Smart, J. E. & Reinherz, E. L. (1984) *Cell* **39,** 261–266.
13. Reinherz, E. L. & Schlossman, S. F. (1980) *Cell* **19,** 821–827.
14. Toribio, M. L., de la Hera, A., Pereira, P. & O. de Landazuri, M. (1984) *J. Immunol.* **134,** 2179–2184.
15. McMichael, A. J., Pilch, J. R., Galfre, G., Mason, D. Y., Fabre, J. W. & Milstein, C. (1979) *Eur. J. Immunol.* **9,** 205–210.
16. Malissen, B., Rebai, N., Liabeuf, A. & Mawas, C. (1982) *Eur. J. Immunol.* **12,** 739–747.
17. UIUS-WHO Nomenclature Subcommittee (1984) *Immunol. Today* **5,** 158–159.
18. Olive, D., Dubreuil, P., Charmot, D., Mawas, C. & Mannoni, P. (1984) in *Proceedings of Leukocyte Conference*, eds. Mitchison, N. A. & Feldmann, M. (Humana, London), in press.
19. Barnstable, C. J., Bodmer, W., Brown, G., Galfre, G., Milstein, C., Williams, A. F. & Ziegler, A. (1978) *Cell* **14,** 9–20.
20. Landegren, U., Ramstedt, U., Axberg, I., Ullberg, M., Jondal, M. & Wigzell, H. J. (1982) *J. Exp. Med.* **155,** 1579–1584.
21. Gelin, C., Boumsell, L., Dausset, J. & Bernard, A. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 4912–4916.
22. Ceredig, R., Sekaly, R. P. & MacDonald, H. R. (1983) *Nature (London)* **303,** 248–250.
23. Chang, T. W., Kung, P. C., Gingras, S. P. & Goldstein, G. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 1805–1808.
24. Platsoucas, C. D. & Good, R. A. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4500–4504.
25. Biddison, W. E., Rao, P. E., Talle, M. A., Goldstein, G. & Shaw, S. (1982) *J. Exp. Med.* **156,** 1065–1076.
26. Hercend, T., Reinherz, E. L., Meuer, S., Schlossman, S. F. & Ritz, J. (1983) *Nature (London)* **301,** 358–360.
27. Snodgrass, H. R., Kisielow, P., Kiefer, M., Steinmetz, M. & von Boehmer, H. (1985) *Nature (London)* **313,** 592–595.
28. Takack, S. L., Osawa, H. & Diamanstein, T. (1984) *Eur. J. Immunol.* **14,** 1152–1156.
29. Habu, S. & Okomura, K. (1984) *Immunol. Rev.* **82,** 117–139.
30. Ceredig, R., Dialynas, D. P., Fitch, F. W. & MacDonald, H. R. (1983) *J. Exp. Med.* **158,** 1654–1671.
31. Kisielow, P., Leiserson, W. & von Boehmer, H. (1984) *J. Immunol.* **133,** 1117–1123.
32. Fowlkes, B. J. (1984) Dissertation (George Washington Univ., Washington, DC).
33. Dialynas, D. P., Loken, M. R., Glasebrook, A. L. & Fitch, F. W. (1981) *J. Exp. Med.* **153,** 595–604.
34. Marrach, P., Endres, R., Schimonkevitz, R., Zlotnik, A., Dialynas, D. P., Fitch, F. W. & Kappler, J. (1983) *J. Exp. Med.* **158,** 1077–1091.
35. Sakaguchi, S., Fukuma, K., Kuribayasi, K. & Masuda, T. (1985) *J. Exp. Med.* **161,** 72–87.
36. DeFreitas, E. C., Vella, S., Linnenbach, A., Zmijewski, C., Koproski, H. & Croce, C. M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6646–6650.
37. Wofsy, D., Hardy, R. R. & Seaman, W. E. (1984) *J. Immunol.* **132,** 2686–2689.