Int. J. Dev. Biol. 36: 573-577 (1992)

Short Contribution

Development of fetal thymocytes in organ culture: effect of corticosteroids

MARGOT A. SKINNER[1]*, LOUISE MOFFATT[1] and STEPHEN J.M. SKINNER[2]

[1]*Department of Molecular Medicine and* [2]*Department of Paediatrics, University of Auckland School of Medicine, New Zealand*

ABSTRACT Corticosteroids affect the development of fetal foregut-derived organs in which epithelial-mesenchymal interactions are associated with the developmental process. The thymus is one such organ and is profoundly sensitive to corticosteroids when mature. In this study corticosterone (CS) effects on fetal thymocyte development were investigated using a fetal thymus organ culture system which allows the growth, differentiation, and function of developing thymocytes to be monitored *in vitro*. CS inhibited, but did not block growth of fetal thymocytes, although the appearance of mature thymocytes was inhibited, similar to previously reported effects of interleukin 2 (IL2). CS enhanced the proportion of Mac1+, Ia+ and FcR+ cells and maintained high levels of IL2 receptor (IL2R) positive immature cells. Functional cytotoxic cells were detected in CS-treated organ cultures which expressed a Thy 1-, CD8- phenotype, atypical for thymus derived killer cells. While this cytotoxicity may be stimulated by CS, it could simply be due to a relative depletion of the main pool of thymocytes. These cytotoxic cells may have a role in directing apoptotic mechanisms occurring during thymocyte development.

KEY WORDS: *apoptosis, thymocytes, corticosteroids, cytotoxicity*

The thymus is the primary site where immunocompetent T lymphocytes differentiate from their precursor cells. During the course of development the genes encoding the T cell receptor are rearranged and expressed for the first time so that the T cells which leave the thymus can recognise antigenic peptides in the context of self major histocompatibility (MHC) antigens with the appropriate affinity (Fowlkes and Pardoll, 1989; Schwarz, 1989; von Boehmer *et al.*, 1989). In the adult thymus, T cells at various stages of development are present. During the developmental process, cell division and differentiation appear to be accompanied by cell death (Scollay and Shortman, 1984). The majority of the cells destined to die within the thymus ($CD4^+$ $CD8^+$) belong to the same population of cortical thymocytes which are sensitive to corticosteroids (Weissman, 1973) and represent approximately 85% of thymocytes. Thymocytes are thought to die by a process termed apoptosis, which is characterized by early changes in the nuclear membrane, followed by rapid degradation of DNA (Sellins and Cohen, 1987). A similar endogenous suicide mechanism is activated by glucocorticoids and by cytotoxic T lymphocytes in target cells (Ucker, 1987).

During fetal thymocyte development there is a sequential appearance of cells at different stages of maturity, beginning with colonisation of the thymus anlage by cells from the fetal liver at about day 11 of mouse embryonic development, which can be followed by cell surface differentiation molecules (Fowlkes and Pardoll, 1989; Jenkinson and Owen, 1990). The fetal thymus organ culture system allows the ontogeny of thymocytes to be studied *in vitro* and is more amenable to experimental manipulation than the thymus in intact animals (Crosier *et al.*, 1989; Jenkinson and Owen, 1990). In previous work we have shown that Thy1+ cytotoxic cells can be detected in fetal thymus organ cultures (FTOC) when the lymphokine IL 2 is present at an age equivalent to day 17 of gestation (Skinner *et al.*, 1987b; Skinner and Marbrook 1988). The presence of cytotoxic cells is accompanied by inhibition of fetal thymocyte growth and differentiation. Here we investigate the effect of corticosterone (CS) on the growth, expression of cell surface differentiation markers and effector function of thymocytes in FTOC.

Fetal thymic lobes were taken from day 14 embryos and placed in organ culture for 10 days with or without CS (10^{-6}M-10^{-9}M). In control cultures the number of viable cells per lobe increased for a period of 4-6 days ($2.9x10^5$ on day 6 of culture), after which time growth ceased and some thymocytes died (Fig. 1A). In FTOC treated with CS

Abbreviations used in this paper: C', complement; CS, corticosterone; FCA, flow cytometric analysis; FTOC, fetal thymus organ culture; IL2, interleukin 2; IL2R, interleukin 2 receptor; MHC, major histocompatibility complex; NK, natural killer; *scid*, severe combined immune deficiency; DN, double negative; DP, double positive; SP, single positive.

***Address for reprints**: Department of Molecular Medicine, School of Medicine, University of Auckland, Private Bag 92019, Auckland, New Zealand. FAX: 64-9-373 5215.

0214-6282/92/$03.00

Printed in Spain

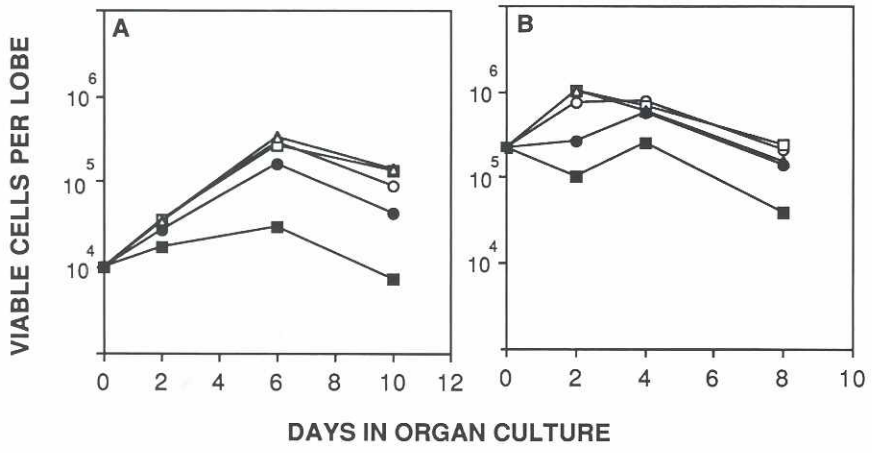

Fig. 1. The effect of CS on the growth of cells in fetal thymic organ cultures. *C57BL/10J thymic lobes from fetal mice at 14* **(A)** *or 16* **(B)** *days of gestation were cultured alone (Δ) or with CS at 10^{-6}M (■), 10^{-7}M (●) 10^{-8}M (○) or 10^{-9}M (❑) and the number of viable cells was estimated at the times indicated. Each point represents the mean of 5 lobes with SD not exceeding 15%.*

(10^{-6}M or 10^{-7}M) a decrease in the apparent cell growth rate was observed. Although the cell numbers increased during the first 6 days of culture, the thymic lobes yielded 10-fold fewer cells when 10^{-6} M CS was present ($3x10^4$ cells per lobe, Fig. 1A). In FTOC from day 16 embryos, viable cells increased during the first 2 days of organ culture (Fig. 1B) whereas with CS (10^{-6}M), viable cells decreased during this time. The effect of 10^{-7} M CS was similar, although not so marked, with a parallel change in the number of viable cells per lobe over the 2-4 day period. CS did not appear to increase dead cell numbers (not shown) but decreased eventual thymocyte numbers.

The effects of CS on the expression of CD4 and CD8 cell surface markers was compared with the effects of IL-2. With IL-2 the growth of CD4- CD8- (double negative, DN) cells was inhibited, as was the generation of CD4+ CD8+ (double positive, DP) cells (Fig. 2). In contrast, with CS the number of DN cells was similar to controls after 6 days FTOC. The major effect of CS was a decrease in the number of cells expressing both CD4 and CD8, although single positive (SP) CD4+ and CD8+ cells were also decreased (Fig. 3). When the expression of a number of other cell surface markers was examined, it was clear that CS treatment enhanced the proportion of cells at 6 days FTOC which expressed Mac1 (from 0% to 14%), Ia (from 8% to 28%) and FcR (from 4% to 36%). It also caused a higher proportion of cells expressing the IL2R to be maintained (Table 1). After exposure to IL2, the proportion of cells expressing Mac1, Ia, FcR or IL2R was not markedly different to controls.

When IL2 is added to FTOC, Thy1+ CD8- cells with cytotoxic activity are detected (Skinner and Marbrook, 1988). To determine if treatment with CS also revealed a cytotoxic population of cells, the cytotoxic activity of fetal thymocytes was analyzed after exposure of FTOC to CS. Cytotoxic activity against P815 target cells was present in the CS-treated lobes (Fig. 4A), but was less potent than that obtained with IL2 treatment. Lysis of the natural killer (NK) sensitive targets YAC-1 was detected in IL2 but not in CS-treated lobes (Fig. 4B). The lytic activity of the cytotoxic cells from CS-treated FTOC was not decreased after incubation with anti-Thy1 or antiCD8 antibody plus complement, indicating a Thy1- CD8- phenotype (Table 2).

In summary, CS inhibited but did not totally block the growth of fetal thymocytes. Mature SP cells were still detectable. There was also an increase in the numbers of cells bearing macrophage markers, and cells with cytotoxic activity were detectable.

Not only do corticosteroids exert several effects on T cells but they are also potent stimuli for mesenchymal/epithelial interactions and differentiation within maturing foregut derived organs (Slavkin *et al.*, 1984). In the rat and other species, there is a marked increase in fetal corticosteroid output in the days immediately preceding birth (Smith *et al.*, 1982). It is thus appropriate to de-

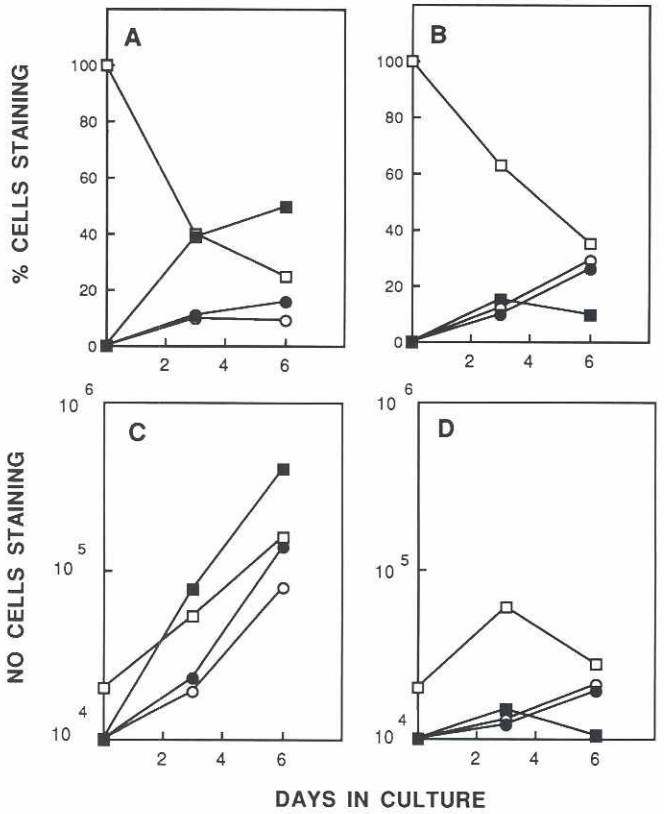

Fig. 2. Effect of IL2 on fetal thymocyte populations. *Two color flow cytometric analysis (FCA) were performed to examine co-ordinate expression of CD4 and CD8 after IL2 (100 units per ml) treatment* **(B,D)** *compared to controls* **(A,C).** *FCA were performed on fetal thymic lobes at day 14 of gestation and after 3 or 6 days' organ culture of these lobes. Data were obtained from the pooled cells of at least 5 thymic lobes. Shown are staining results for CD4-CD8- (❑), CD4+CD8+ (■), CD4-CD8+ (○) and CD4+CD8- (●) populations. In A and B the results are expressed as % cells staining, taking into account that CD4-CD8- cells do not stain. In C and D the number of cells per thymic lobe belonging to each of the four populations is shown.*

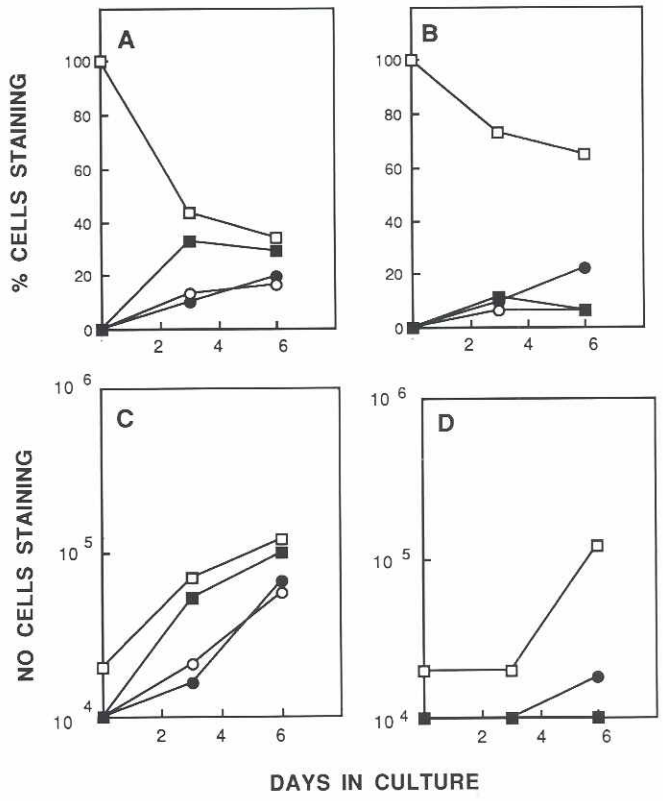

Fig. 3. Effect of CS on fetal thymocyte populations. *Two color FCA were used to examine the co-ordinate expression of CD4 and CD8 after CS (10^{-6}M) treatment* **(B,D)** *compared to controls* **(A,C)**. *FCA were performed on fetal lobes at 14 days of gestation and after 3 or 6 days in organ culture and data were obtained from the pooled cells of at least 5 thymic lobes. Shown are staining results of the four populations CD4-CD8- (❑), CD4+CD8+ (■), CD4-CD8+ (○) and CD4+CD8- (●). In A and B results are expressed as % of cells staining in each population, whereas in C and D results are expressed as the number of cells per lobe in each population.*

TABLE 1

PHENOTYPE OF CELLS FROM FTOC AFTER EXPOSURE TO CS

Cell surface molecule	Treatment of FTOC: Control**		CS**		IL2*	
	Percentage of positively staining cells					
	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6
Thy1	91	89	87	84	91	88
Ly1	69	61	69	71	55	77
Mac1	0	0	5	14	0	0
IL2R	59	30	49	40	49	26
Ia	12	8	10	28	8	10
FcR	6	4	6	36	4	4

Results are % of cells expressing a particular cell surface antigen as measured by monoclonal antibody staining and flow cytometry. Cells are from control fetal thymic cultures, or ones exposed to 10^{-6}M CS or 100 units per ml IL2

*One representative experiment of two. **One representative experiment of three.

termine the effects of CS on the developing thymus since this is a foregut derived structure with well differentiated functions for both the epithelium and the mesenchymal derived lymphoid cells. The effect of CS on the adult thymus is well documented with the depletion of cortical thymocytes (Weissman, 1973), of distinct subpopulations of medullary thymocytes (Chen *et al.*, 1982; van Vliet *et al.*, 1986) and changes in MHC antigen expression and morphology of the cortical reticular epithelial stroma (van Vliet *et al.*, 1986).

The effect of corticosteroids on the fetal thymus has not been so rigorously studied. Using the FTOC system, typical small cortical thymocytes were eliminated after exposure to hydrocortisone and a relative enrichment of Thy1- Ia+ cells was found (Mandel and Scollay, 1981; Barr *et al.*, 1984). However, FTOC were equivalent to at least 17 days of gestation before corticosteroid was added. In this study the effects of CS on fetal thymocyte development from day 14 of gestation is compared to the effects of IL2, a T cell growth factor previously shown to inhibit fetal thymocyte development (Skinner *et al.*, 1987b). Addition of IL2 to 14 day FTOC inhibited the normal growth of DN thymocytes as well as the differentiation of DN to DP cells, and the progression to SP cells (Fig. 2). In contrast, CS did not totally inhibit the growth of DN cells (Fig. 3) and thus cannot simply be considered a thymolytic agent within the developing fetal thymus. In addition the differentiation pattern was changed, with a marked decrease in DP cells and a relative enrichment for cells expressing Mac1, Ia, and FcR (Table 1). CS has presumably depleted the main lymphoid cell group, allowing for an enrichment of early T cells and non-lymphoid cells. IL2 also caused a decrease in DP cells, but without enriching for Mac1+, Ia+ or FcR+ cells, suggesting this may be a unique effect of corticosteroids.

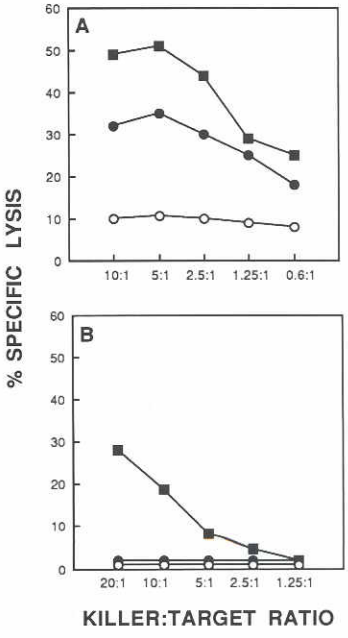

Fig. 4. Comparison of cytototoxic activity in fetal thymic lobes cultured with CS or IL2. *Thymic lobes from 14 day fetal mice were cultured with CS at 10^{-6}M (●), IL2 at 100u/ml (■) or without treatment (○) for 6 days. The cells were harvested and assayed for cytotoxic activity in a 4hr ^{51}Cr release assay using P815* **(A)** *or YAC-1* **(B)** *target cells. The SD of triplicate determinations did not exceed 3% specific lysis.*

TABLE 2

PHENOTYPIC ANALYSIS OF CYTOTOXIC CELLS FROM CS TREATED FETAL THYMIC LOBES

Antibody and /or complement	%Specific Lysis	SD
-C'	16	2.7
Anti Thy1	13.7	2.7
Anti Thy1+C'	13.0	3.9
anti-CD8	19.9	5.9
anti CD8+C'	18.9	4.7

Cells from thymic lobes cultured with 10^{-6}M CS were incubated with C', antibody or antibody plus C' and then assayed for cytolytic activity on ^{51}Cr-labeled P815 target cells at a killer-to-target ratio of 4:1. The results from triplicate assays are shown with SD.

Exposure of thymic organ cultures to CS allowed the detection of Thy1- CD8- cytotoxic cells (Fig. 4, Table 2). The question arises as to whether this is due to depletion of other cells which might inhibit or dilute out cytotoxic cells or is due to a direct effect of CS on cells with cytotoxic potential. A similar conundrum exists with the Thy1+ CD8- cytotoxic cells present in thymic lobes treated with IL2 (Skinner and Marbrook, 1988). IL2, however, is known to induce or enhance cytotoxic activity in lymphoid populations (Grimm *et al.*, 1982; Skinner *et al.*, 1987a). No such effect has previously been reported for CS. Cytotoxic cells or their inactive precursors are present in the thymus (Ballas and Rasmussen, 1987; Michon *et al.*, 1988) particlarly in *scid* mice where the thymus is hypocellular and contains only immature T cells, (Garni-Wagner *et al.*, 1990). The early T cell precursors present in *scid* mice have a similar phenotype to CS-induced killers but they mediate NK activity. No NK activity could be detected after CS treatment (Fig. 4). Alternatively, these cytotoxic cells may belong to the macrophage lineage, particularly as Mac1+ cells are enriched in FTOC after exposure to CS (Table 1).

If cytotoxic cells, unmasked by CS treatment, are present in the normal fetal thymus, do they have a role in T cell development? It is thought that bone marrow-derived cells, belonging not to the T cell lineage but to the macrophage lineage, are involved in the "negative" selection process in the thymus which ensures that developing autoreactive T cells are eliminated (Schwarz, 1989; van Ewijk, 1991). Directed cell death may be a feature of intrathymic "positive" selection (McDonald and Lees, 1990) and molecular mechanisms for the clonal deletion of autoreactive cells have been proposed (Smith *et al.*, 1989) including the *Fas* antigen that mediates apoptosis (Watanabe-Fukunaga *et al.*, 1992). It remains to be seen which cells within the thymus possess the ligand for the *Fas* antigen. The possibility that the cytotoxic cells detected in this study direct apoptosis within the thymus and are involved in the ability of the thymus to eliminate autoreactive clones is intriguing and merits further investigation.

Experimental Procedures

Mice

Male and female C57BL/10J mice were obtained from breeding colonies maintained in the Cancer Laboratories, School of Medicine, University of Auckland. Embryos were obtained from timed matings with the day after an 18hr exposure of females to males being designated day 0.

Lymphokine

Human recombinant IL2 was kindly supplied by Immunex Corporation, Seattle. Units of IL2 activity were determined in an IL2-dependent T cell growth assay where one unit of activity was defined as the amount required to stimulate 50% of the maximal growth response.

Fetal thymus organ cultures

Intact fetal thymic lobes were cultured as described previously (Skinner *et al.*, 1987b). Briefly 10-15 lobes were placed on Millipore filters (0.45μm) supported by 1cm squares of gelatin sponge (Gelfoam, Upjohn, Kalamazoo USA). and placed in a dish of culture medium (Iscoves modified Dulbecco's medium supplemented with 10% fetal calf serum, FCS and glutamine 0.2mg/ml). Cultures were incubated in a humidified incubator at 37°C containing 10% CO_2 in air. After culture, thymocytes were released, layered over FCS and washed twice in culture medium. Viable cells were assessed by using trypan blue exclusion.

Cell staining and flow cytometry

To detect expression of CD4 and CD8 antigens, directly conjugated GK1.5-phycoerythrin and fluoresceinated 53-6.7 antibodies were used (Becton Dickinson, Mountain View, CA). Other unconjugated monoclonal antibodies were as previously described (Skinner *et al.*, 1989) and were used in conjunction with a second goat anti-mouse fluoresceinated IgG or IgM (Becton Dickinson). Cells were incubated at 4°C in culture medium containing 0.01% sodium azide and monoclonal antibody for 20 min followed by two washes. Flow cytometry was performed on a fluorescence-activated cell sorter (FACS 440, Becton Dickinson). A minimum of 10,000 cells were analyzed for each sample. Dead cells were excluded from analysis by electronic gating of cells staining with propidium iodide.

Antibody plus complement treatment

Cells were harvested from organ cultures and incubated with the mouse anti-CD8 antibody 53-6.7 (Ledbetter and Herzenberg, 1979) or anti-Thy1, T2431.7, (Dennert *et al.*, 1980) at 37°C for 30 min at an appropriate concentration (Skinner and Marbrook, 1988). Guinea pig complement (C') absorbed with adult thymocytes was added at a final concentration of 1:30; the cells were incubated for a further 30 min at 37°C and then washed twice.

Cytotoxicity assay

Cytotoxicity was measured by a standard ^{51}Cr-release assay with minor modifications as previously described (Skinner *et al.*, 1987b). Target cells were mastocytoma P815 ($H2^{d}$) and the mouse NK sensitive target cell YAC-1. Percent specific lysis was determined by the formula:

$$\% \text{ specific lysis} = \frac{\text{experimental cpm - spontaneous cpm}}{\text{maximum uptake cpm-spontaneous cpm}} \times 100$$

Standard deviations of triplicate determinations were generally less than 3% of the specific lysis.

Acknowledgments

This work was supported in part by the Auckland Medical Research Foundation and the Health Research Council of New Zealand.

References

BALLAS, Z. K. and RASMUSSEN W. (1987). Lymphokine-activated killer (LAK) cells. III. Characterization of LAK precursors and susceptible target cells within the murine thymus. *J. Immunol. 139:* 3542-3549.

BARR, I.G., PYKE, K.W., PEARCE, P., TOH, B.-H. and FUNDER, J.W. (1984). Thymic sensitivity to sex hormones develops post-natally; an *in vivo* and an *in vitro* study. *J. Immunol. 132:* 10951099.

CHEN, W.-F., SCOLLAY, R. and SHORTMAN, K. (1982). The functional capacity of thymus subpopulations: limit-dilution analysis of all precursors of cytotoxic lymphocytes and of all T cells capable of proliferation in subpopulations separated by the use of peanut agglutinin. *J. Immunol. 129:* 18-24.

CROSIER, P.S., SKINNER, M., LE GROS, G., HOLDAWAY, K. and WATSON, J.D. (1989). Growth of fetal thymocytes in organ culture: effect of recombinant lymphokines on thymocyte maturation. *Growth Factors 1:* 299-310.

DENNERT, G., HYMAN, R., LESLEY, J. and TROWBRIDGE, I.S. (1980). Effect of cytotoxic monoclonal antibody specific for T200 glycoprotein on functional lymphoid cell populations. *Cell. Immunol. 53:* 350-364.

FOWLKES, B.J. and PARDOLL, D.M. (1989). Molecular and cellular events of T cell development. *Adv. Immunol. 44:* 207-264.

GARNI-WAGNER, B.A., WITTE, P.L., TUTT, M.M., KUZIEL, W.A., TUCKER, P.W., BENNETT, M. and KUMAR, V. (1990). Natural killer cells in the thymus. Studies in mice with severe combined immune deficiency. *J. Immunol. 144:* 796-803.

GRIMM, E.A., MAZUMDER, A., ZHANG, H.Z. and ROSENBERG, S.A. (1982). Lymphokine-activated killer cell phenomenon. Lysis of natural killer resistant and fresh solid tumour cells by interleukin2 activated autologous human peripheral blood lymphocytes. *J. Exp. Med. 155:* 1823-1841.

JENKINSON, E.J. and OWEN, J.J.T. (1990). T cell differentiation in thymus organ cultures. *Semin. Immunol. 2:* 51-58.

LEDBETTER, J.A. and HERZENBERG, L.A. (1979). Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev. 47:* 63-90.

MANDEL, T.E. and SCOLLAY, R. (1981). Differentiation of T lymphocytes in organ culture of fetal mouse thymus: analysis of cells using monoclonal antibodies and flow cytometry. In *The Immune System*, Vol. 2 (Eds. C.M. Steinberg and I. Lefkovits). Karger, Basel, pp. 38-44.

McDONALD, H.R. and LEES, R.K.(1990). Programmed cell death of autoreactive thymocytes. *Nature 343*: 642-644.

MICHON, J.M., CALIGIURI, M.A., HAZANOW, S.M., LEVINE, H., SCHLOSSMAN, S.F. and RITZ, J. (1988). Induction of natural killer effectors from human thymus with recombinant IL-2. *J. Immunol. 140:* 3660-3667.

SCHWARZ, R.H. (1989). Acquisition of immunologic self tolerance. *Cell 57:* 1073-1081.

SCOLLAY, R. and SHORTMAN, K. (1984). Cell traffic in the adult thymus: cell entry and exit, cell birth and death. In *Recognition and Regulation in Cell Mediated Immunity* (Eds. J.D. Watson and J. Marbrook). Marcel Dekker Inc., New York and Basel, pp. 3-30.

SELLINS, K.S. and COHEN, J.J. (1987). Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. *J. Immunol. 139:* 3199-3206.

SKINNER M. and MARBROOK, J. (1988). Effect of interleukin 2 on fetal thymocytes in organ cultures: generation of lymphokine-activated killer cells. *Cell. Immunol. 112:* 104-111.

SKINNER M., MOFFATT L. and MARBROOK J. (1989). Development of thymocytes in organ culture: migrant cells with natural killer cell characteristics. *Immunol. Cell. Biol. 67:* 107-114.

SKINNER, M., ERTL, H.C.J. and FINBERG, R.W. (1987a). Lymphokines induce specificity degradation in virus-induced cytolytic T-cell clones. *Cell. Immunol. 109:* 159-168.

SKINNER, M., LE GROS, G., MARBROOK, J. and WATSON, J.D. (1987b). Development of fetal thymocytes in organ cultures. Effect of interleukin 2. *J. Exp. Med. 165:* 1481-1488.

SLAVKIN, H.C., SNEAD, M.L. ZEICHNER-DAVID M., JASKOLL, T.F. and SMITH, B.T. (1984). Concepts of epithelial-mesenchymal interactions during development: tooth and lung organogenesis. *J. Cell. Biochem. 26:* 117-125.

SMITH B.T., TANSWELL A.K., MINSHALL D., BOGUES, W.N. and VREEKEN E. (1982). Influence of corticosteroids on glycogen content and steroid 11-reductase activity in lung and liver of the fetal and newborn rat. *Biol. Neonate 42:* 201-207.

SMITH, C.A, WILLIAMS G.T., KINGSTON, R., JENKINSON E.J. and OWEN, J.J.T. (1989). Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature 337:* 181-184.

UCKER, D.S. (1987). Cytotoxic T lymphocytes and glucocorticoids activate an endogenous suicide pathway process in target cells. *Nature 372:* 62-64.

VAN EWIJK, W. (1991). T-cell differentiation is influenced by thymic microenvironments. *Annu. Rev. Immunol. 9:* 591-615.

VAN VLIET, E., MELIS, M. and van EWIJK, W. (1986). The influence of dexamethasone treatment on the lymphoid and stromal composition of the mouse thymus: a flowcytometric and immunohistological analysis. *Cell. Immunol. 103:* 229-240.

VON BOEHMER, H., TEH, H.S. and KISIELOW, P. (1989). The thymus selects the useful and destroys the harmful. *Immunol. Today 10:* 57-61.

WATANABE-FUKUNAGA, R., BRANNAN, C.I., COPELAND, N.G., JENKINS, N.A. and NAGATA, S. (1992). Lymphoproliferative disorder in mice explained by defects in *Fas* antigen that mediates apoptosis. *Nature 356:* 314-317.

WEISSMAN, I.L. (1973). Thymus cell maturation. Studies on the origin of cortisone resistant thymic lymphocytes. *J. Exp. Med. 137:* 504-510.

Accepted for publication: September 1992