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Developmental changes predispose the fetal thymus to positive selection of $CD4^+ CD8^-$ T cells

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SUMMARY

Selection of a competent T-cell repertoire is dependent on complex interactions between immature thymocytes and components of the thymic stroma. These events may be preserved *in vitro* by excising developing thymus rudiments and maintaining them under carefully controlled conditions in fetal thymus organ cultures (FTOC). Using this approach, we have shown that the ability of C57Bl/6 thymi to sustain positive selection of mature $CD4^+ CD8^-$ cells is profoundly influenced by the day of gestation on which they are excised: while thymocytes from day 14 rudiments fail to progress beyond the $CD4^+ CD8^+$ stage of the developmental pathway, day 15 and day 16 thymi support the differentiation of $CD4^+ CD8^-$ thymocytes. Importantly, day 16 thymocytes transferred to day 14 deoxyguanosine-treated rudiments are likewise arrested at the $CD4^+ CD8^+$ stage, suggesting that the thymic microenvironment of day 14 rudiments, rather than the state of differentiation of the thymocytes they contain, is responsible for the block in positive selection. Our studies of the stromal elements of day 14 rudiments have, however, revealed no obvious deficiencies in the cell types represented, or their expression of class II major histocompatibility complex (MHC) determinants. Furthermore, we have been unable to circumvent the blockage in positive selection by the addition of certain cytokines expressed late during gestation. These results suggest that subtle changes occurring at day 15 of ontogeny render the thymic microenvironment capable of positive selection.

INTRODUCTION

The integrity of the peripheral T-cell repertoire is dependent on thymic selection events occurring early during ontogeny. While cells expressing T-cell receptors (TCR) with high affinity for self-determinants are actively deleted or silenced, others are rescued from programmed cell death by the process of positive selection.[1] Although the exact nature of this process remains obscure, the consequences of positive selection are well defined: mature T cells show commitment to either the $CD4^- CD8^+$ or $CD4^+ CD8^-$ lineage, reflecting their specificity for antigen in the context of class I or class II products of the major histocompatibility complex (MHC), respectively.[2,3]

In order to understand the mechanisms involved in bringing about these events, strategies have been devised to promote selection under carefully controlled conditions *in vitro*. Accordingly, thymic rudiments maintained in fetal thymus organ culture (FTOC) have been shown to faithfully emulate T-cell selection *in vivo*.[4] Here, however, we report that the ability of fetal thymi to support positive selection of $CD4^+ CD8^-$ T cells is entirely dependent on the day of ontogeny on which they are excised. These findings suggest that developmental changes occurring within the thymic microenvironment at day 15 of gestation are critical for positive selection of a mature class II-restricted T-cell repertoire.

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Abbreviations: β_2m, β_2-microglobulin; DC, dendritic cell; dGuo, 2′-deoxyguanosine; FTOC, fetal thymus organ culture; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL, interleukin; Mϕ, macrophage; UEA-1, *Ulex europaeus* agglutinin-1.

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MATERIALS AND METHODS

Animals

C57Bl/6 and C3H/He mice were obtained from Harlan Olac (Bicester, UK) and maintained under specific pathogen-free (SPF) conditions at the Sir William Dunn School of Pathology (University of Oxford, Oxford, UK). Timed matings of C57Bl/6 mice were performed such that the day on which a vaginal plug was observed was designated day 0 of gestation.

FTOC

Thymus rudiments were dissected from embryos and maintained in hanging drop cultures, as described previously.[4] In some experiments, RPMI-1640 medium containing 10% fetal

calf serum (FCS; R10) was further supplemented with 50% tissue culture supernatant from clones B21-2 (anti-I-A^{bd}; rat γ2b) or 10-2.16 (anti-I-A^{krfsu}; mouse γ2b). On such occasions, thymi were transferred to fresh hanging drops every 2 days to prevent exhaustion of the medium.

In order to deplete fetal thymi of endogenous thymocytes, rudiments were cultured in R10 supplemented with 1·35 mM 2′-deoxyguanosine (dGuo; Sigma, Poole, UK).[5] Thymocytes for recolonization of alymphoid rudiments were prepared by incubating day 16 fetal thymi in R10 containing 1 mg/ml collagenase (Sigma) for 25 min. Rudiments were disrupted, the liberated cells washed in R10 and plated onto 35-mm tissue culture plates. After incubating for 1 hr to promote adherence of macrophages (Mϕ) and dendritic cells (DC), non-adherent thymocytes were collected and 4×10^4 cells introduced into hanging drops containing single alymphoid rudiments. After culturing for 2 days, recolonized thymi were transferred to fresh hanging drops and cultured for a further 5 days.

Cytokines

Recombinant granulocyte–macrophage colony-stimulating factor (rGM-CSF; Genzyme, West Malling, UK) was used at a concentration of 10 ng/ml. Recombinant interleukin-1α (rIL-1α; a kind gift from Professor S. Gordon, Sir William Dunn School of Pathology) was added to FTOC at a final concentration of 100 U/ml, while rIL-2 (a kind gift from Dr M. Dallman, Nuffield Dept of Surgery, John Radcliffe Hospital, Oxford, UK) was employed at 10–100 U/ml.

IL-3 was obtained by harvesting supernatant from cultures of WEHI-3b cells and was assayed for its ability to support the differentiation of multiple colonies from the bone marrow of C57Bl/10 mice. Complete medium supplemented with 4% WEHI-3b supernatant was found to be optimal and was therefore used at this concentration in FTOC.

Flow cytometry

Thymocytes isolated from FTOC were analysed for expression of CD4 and CD8 by staining with biotinylated YTS191.1 (anti-CD4; rat γ2b) and fluorescein isothiocyanate (FITC)-conjugated YTS169.4 (anti-CD8; rat γ2b). Binding of YTS191.1 was visualized by the addition of streptavidin–phycoerythrin (Becton Dickinson, Mountain View, CA). Ten thousand events from each sample were gated by forward and side scatter, and analysed using either a Becton Dickinson FACScan or FACSort interfaced with a Hewlett-Packard 310 computer.

Mixed leucocyte reaction

Freshly excised day 14 rudiments from C57Bl/6 mice (H-2^b) were used as a source of stimulators. Unfractionated cells were washed and irradiated with 2000 rads from a ^{137}Cs source. Responder T cells were purified from C3H/He (H-2^k) spleens by passage of unfractionated splenocytes over nylon wool, as described elsewhere.[6] Stimulator populations were titrated into cultures of responder T cells from a top ratio of 1 : 1. After a 4-day incubation, cultures were pulsed for 18 hr with 0·5 μCi/well of [^{3}H]thymidine ([^{3}H]TdR; Du Pont, Stevenage, UK), harvested and counted using a flat-bed scintillation counter (Pharmacia-Wallac, St Albans, UK).

Immunohistochemistry

Frozen sections 7 μm thick were depleted of endogenous peroxidase activity as described elsewhere[7] and the following monoclonal antibodies (mAb) applied as tissue culture supernatants: B21-2 (anti-I-$A^{b,d}$; rat γ2b), F4/80 (anti-Mϕ; rat γ2b) and 4F1E4 (anti-cortical epithelium; rat μ)[8] (a kind gift from Professor M. Ritter, RPGMS, London, UK). Binding was detected using horseradish peroxidase (HRP)-conjugated rabbit anti-rat antiserum (Dako, High Wycombe, UK) followed by 0·5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (Sigma) containing 0·8% H_2O_2 (v/v). Medullary epithelium was visualized by binding of the lectin *Ulex europaeus* agglutinin 1 (UEA-1; Dako) followed by HRP-conjugated rabbit anti-UEA-1 (Dako). Sections were counterstained with Harris' haematoxylin (BDH, Poole, UK).

RESULTS

Failure of day 14 fetal thymi to support positive selection of $CD4^+$ $CD8^-$ T cells

While devising a strategy for the study of positive selection *in vitro*, we investigated whether the differentiation potential of thymocytes developing in organ cultures was influenced by the day of gestation on which the thymi were excised. Accordingly, thymic rudiments from C57Bl/6 embryos at days 14, 15 and 16 of gestation were cultured in parallel for 7 days. Phenotypic analysis of thymocytes from day 16 rudiments revealed the majority to express neither CD4 nor CD8 prior to the onset of culture (Fig. 1e). After 7 days in FTOC, however, a substantial proportion had progressed to the $CD4^+$ $CD8^+$ stage, while a discrete $CD4^+$ $CD8^-$ subset had formed representing 10·9% (Fig. 1f). Although thymocytes from day 15 fetal thymi were almost exclusively $CD4^-$ $CD8^-$ precursors at the start of organ culture (Fig. 1c), a $CD4^+$ $CD8^-$ population was likewise observed at day 7 (Fig. 1d). In contrast, $CD4^-$ $CD8^-$ thymocytes from day 14 rudiments developed a substantial $CD4^+$ $CD8^+$ subset, but were arrested at this stage of differentiation, consistently failing to generate mature $CD4^+$ $CD8^-$ cells (Fig. 1b).

In order to determine whether 7 days was sufficient for positive selection of thymocytes to become apparent, day 14 rudiments were maintained in FTOC for either 14 or 21 days. Analysis of the CD4/CD8 profile failed to detect any increase in the number of $CD4^+$ $CD8^-$ thymocytes with time (Fig. 2).

We next addressed the possibility that the $CD4^+$ $CD8^-$ subset from day 15 and day 16 rudiments had resulted from the accumulation of functionally immature $CD4^+$ $CD8^-$ cells,[9] rather than reflecting the outcome of positive selection. Such immature precursors represent an intermediate step in the generation of the $CD4^+$ $CD8^+$ cells from $CD4^-$ $CD8^-$ thymocytes:[10] as they lack expression of functional TCR, however, their generation is independent of the TCR–MHC interactions fundamental to positive selection.[11] We therefore investigated whether targeting class II on thymic epithelium with a mAb known to sterically hinder ligation of the TCR, would inhibit development of the $CD4^+$ $CD8^-$ subset. Fetal thymi from day 16 embryos were therefore cultured for 7 days in medium containing supernatant from clone B21-2 (anti-I-A^{bd}) or control supernatant from clone 10-2.16 (anti-I-A^{krfsu}). While a discrete $CD4^+$ $CD8^-$ population was detectable in control rudiments after 7 days (Fig. 3a), their

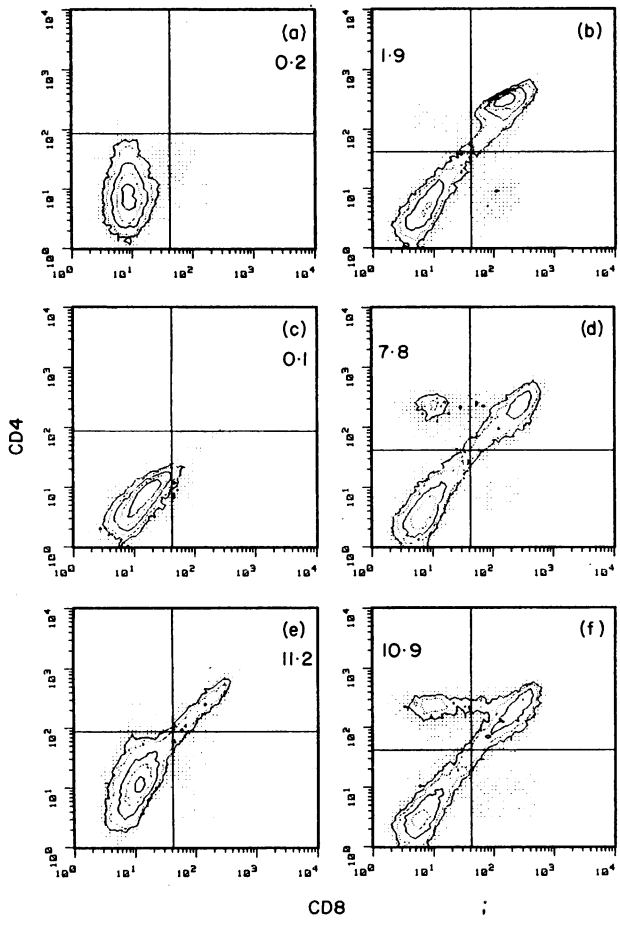

Figure 1. Thymocyte differentiation in FTOC. Surface expression of CD4 and CD8 by thymocytes from rudiments excised at days 14 (a, b), 15 (c, d) and 16 (e, f) of gestation either prior to FTCC (a, c and e), or after culturing for 7 days (b, d and f).

development was completely inhibited in the presence of B21-2 (Fig. 3b). These results confirm that while day 15 and day 16 rudiments support the positive selection of mature T cells, thymi excised at day 14 of gestation fail to do so, the developmental pathway of thymocytes becoming arrested at the $CD4^+$ $CD8^+$ stage.

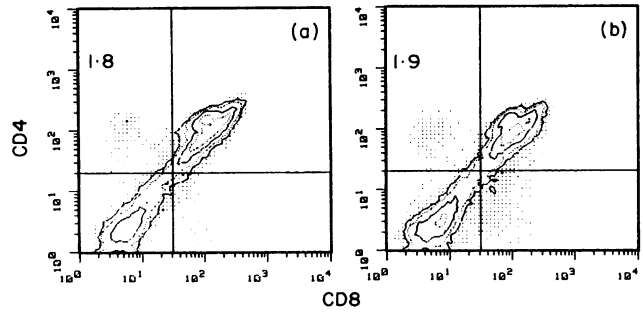

Figure 2. CD4/CD8 profile of thymocytes from day 14 rudiments maintained in FTOC for 14 (a) or 21 days (b).

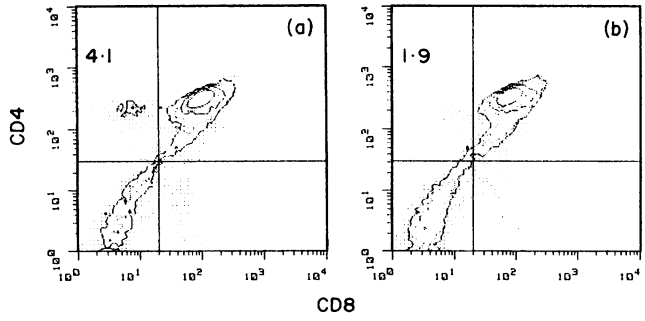

Figure 3. Differentiation of day 16 H-2^b thymocytes in undisrupted thymi cultured for 7 days in supernatant from clone 10.2-16 (anti-I-A^k) (a) or B21-2 (anti-I-A^b) (b).

The microenvironment of day 14 rudiments is responsible for the block in positive selection

To determine whether the block in positive selection could be attributed to the developmental stage of day 14 thymocytes or was a function of the microenvironment in which they developed, day 14 rudiments were cultured for 5 days in 1·35 mM dGuo to deplete endogenous thymocytes, leaving stromal elements intact.[5] The resulting alymphoid rudiments were recolonized with thymocytes from day 16 thymi, already known to be capable of generating a mature $CD4^+$ $CD8^-$ subset in FTOC (Fig. 1f). In spite of their differentiation potential, such thymocytes failed to undergo significant positive selection (Fig. 4a). In contrast, control rudiments from day 16 embryos recolonized with day 16 thymocytes supported development of a substantial $CD4^+$ $CD8^-$ population (Fig. 4b) expressing high levels of CD3, indicative of

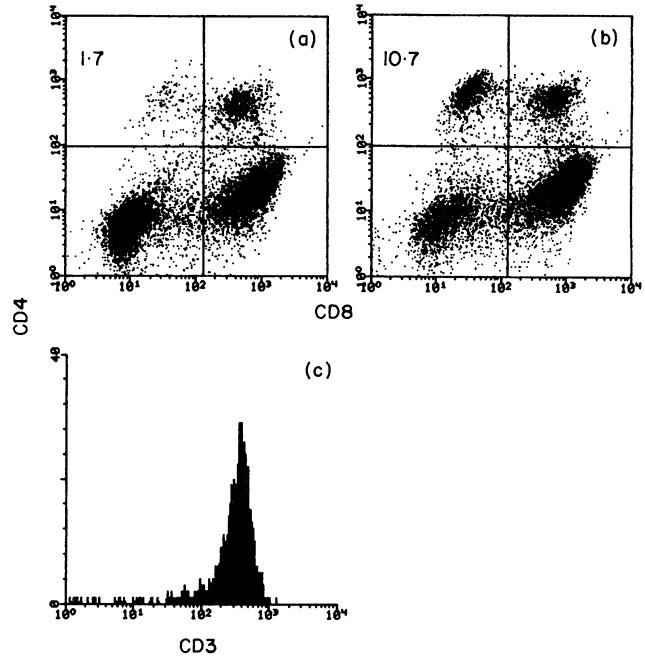

Figure 4. Differentiation of day 16 thymocytes following recolonization of day 14 (a) or day 16 alymphoid rudiments (b). (c) Surface expression of CD3 by $CD4^+$ $CD8^-$ thymocytes from (b).

positive selection having occurred (Fig. 4c). Furthermore, day 14 thymocytes introduced into day 16 alymphoid rudiments likewise gave rise to a discrete $CD4^+$ $CD8^-$ subset (data not shown). These results suggest that the microenvironment afforded by day 14 fetal thymi, rather than the developmental stage of the thymocytes they contain, is responsible for the blockage in positive selection.

Characterization of the microenvironment of day 14 fetal thymi

In attempting to identify the deficiency in the microenvironment of day 14 thymi, we characterized various stromal elements by immunocytochemistry. As cortical epithelium has been widely implicated in positive selection of developing thymocytes,[1] sections from day 14 rudiments were stained to ascertain whether epithelial cells had yet differentiated into cortex and medulla. Staining with 4F1E4[8] revealed a reticular pattern of cortical epithelium with occasional unstained areas of putative medulla (Fig. 5a). The identity of these areas was confirmed by staining alternative sections with the lectin UEA-1, specific within the thymus for α-linked L-fucose residues expressed exclusively by medullary epithelium (Fig. 5b).[12]

Because the lack of $CD4^+$ $CD8^-$ cells in cultures of day 14 thymi was reminiscent of the pattern of differentiation observed in class II-deficient mice,[13] we next investigated whether the thymic epithelium was capable of expressing class II MHC at such an early stage of ontogeny. Sections stained with B21-2 revealed widespread expression of $I\text{-}A^b$ (Fig. 5c). Furthermore, day 14 rudiments cultured in dGuo showed marked up-regulation of class II expression (Fig. 5d) relative to controls cultured in its absence (Fig. 5e). The failure of recolonized alymphoid rudiments to positively select day 16 thymocytes in spite of the abundance of an appropriate restriction element, suggests that the defect in positive selection is unlikely to lie with the thymic epithelium. Interestingly, the similar up-regulation of class I determinants following exposure to dGuo, suggests an explanation for the over-selection of $CD4^-$ $CD8^+$ cells by recolonized fetal thymi observed in earlier experiments (Fig. 4a, b).

Although various experimental approaches have failed to define a role for bone marrow-derived Mϕ and DC in positive selection,[4,14,15] recent studies using β_2-microglobulin (β_2m)-deficient mice have implicated haemopoietic elements in the development of mature $CD4^-$ $CD8^+$ T cells.[16] These findings have suggested that thymic selection events are more permissive than once thought.[17] We therefore assessed the contribution to the day 14 thymic stroma of bone marrow-derived elements. Sections stained for the Mϕ-specific marker F4/80, revealed a random distribution of Mϕ throughout the thymus (Fig. 5f). Furthermore, when used as a source of stimulators in an allogeneic mixed lymphocyte reaction (MLR), day 14 rudiments elicited a strong primary response (Fig. 6) thereby confirming previous reports of the presence of DC within the developing thymus as early as day 14 of gestation.[18]

Exogenously added cytokines fail to overcome the blockage in positive selection

In the absence of any apparent defect in the stromal elements of day 14 fetal thymi, we next investigated whether disruption of the normal pattern of cytokine gene expression might account for our observations. Various cytokines and their receptors have been detected during thymic ontogeny in the mouse, and are expressed in two waves: while many are present continuously from day 14 to day 20, expression of IL-1α, IL-2, IL-3 and GM-CSF has been shown to be delayed until day 16 of gestation.[19] We reasoned, therefore, that day 14 rudiments maintained under suboptimal conditions in FTOC, may fail to synthesize one or more of this second wave of cytokines. Consequently, we determined whether supplementing cultures with physiological concentrations of IL-1α, IL-2, IL-3 or GM-CSF would reverse the defect in thymocyte differentiation.

Thymi cultured for 7 days in either rIL-1 or IL-3 yielded CD4/CD8 profiles largely indistinguishable from those of control rudiments (data not shown). While the addition of either rIL-2 or rGM-CSF had no discernable effect on development of the $CD4^+$ $CD8^-$ subset, both cytokines prompted the appearance of a discrete $CD4^-$ $CD8^+$ population, representing up to 13·7% of the total (Fig. 7b–c). Three-colour flow cytometry revealed this population to express a $CD3^-$ $J11d^+$ phenotype (data not shown), implying that such cells represent the accumulation of an immature subset intermediate in the development of $CD4^+$ $CD8^+$ cells from $CD4^-$ $CD8^-$ precursors.[10] Thus, the addition of rIL-2 and rGM-CSF to FTOC actively retarded, rather than promoting, thymocyte differentiation.

DISCUSSION

Our results demonstrate that excision of the thymus from C57Bl/6 embryos at day 14 of gestation inhibits the selection of a mature $CD4^+$ $CD8^-$ T-cell repertoire during FTOC. In contrast, delaying excision by as little as 24 hr permits subsequent differentiation to proceed normally. Although others have hinted at similar findings,[20] some strain specificity appears likely since day 14 rudiments from PL/J mice consistently yield a small but discrete $CD4^+$ $CD8^-$ subset *in vitro* (P.J.F., unpublished observations), findings similar to those reported for BALB/c mice.[21] Nevertheless, these findings suggest that day 14 C57Bl/6 rudiments lack some factor essential for positive selection: we have characterized the microenvironment afforded by such rudiments in an attempt to identify the nature of this deficiency.

Although we cannot rule out the possibility that a critical cell type is excluded from day 14 rudiments, bone marrow-derived stem cells have been shown to colonize the thymus as early as day 11 of ontogeny.[22] In support of this contention, our experiments revealed the presence of a normal contingent of Mϕ and DC by day 14 of gestation.

While the extent of the contribution made by haemopoietic elements to positive selection remains controversial,[1,17,23] the central role played by class II^+ cortical epithelium is largely undisputed, having received support from recent studies of reaggregated fetal thymi maintained in FTOC.[24,25] Nevertheless, our immunocytochemical analysis of the thymic epithelium revealed that differentiation into cortex and medulla had already occurred by day 14, as had widespread expression of class II, suggesting that epithelium is required, but is not sufficient, for positive selection to proceed. These findings have been endorsed by our experiments involving

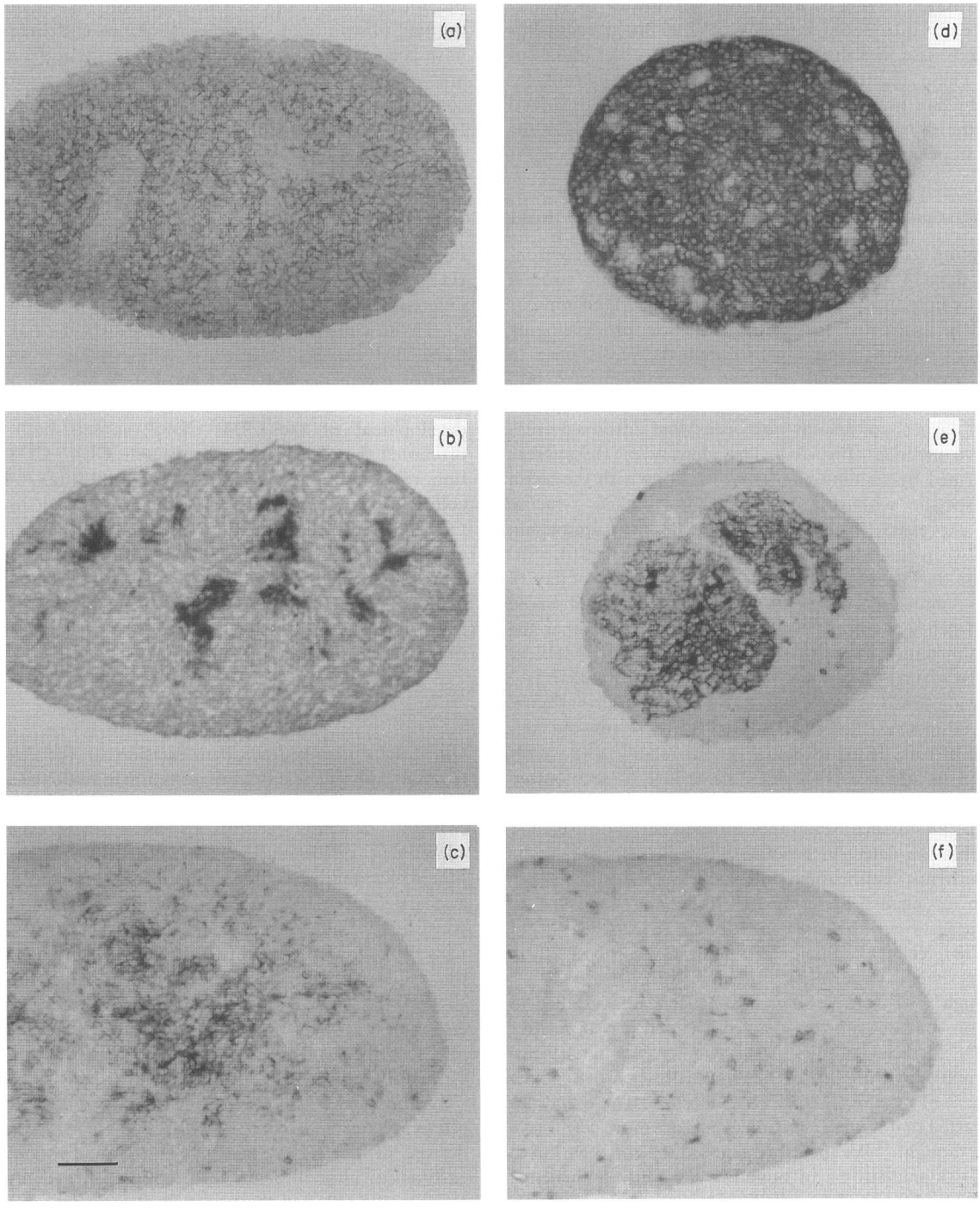

Figure 5. (a–e) Immunocytochemistry of the thymic epithelium of day 14 fetal thymi. (a–c) Sections from freshly excised rudiments stained for cortical epithelium (a), medullary epithelium (b) and class II MHC (c). (d, e) Class II expression by day 14 rudiments cultured for 7 days in dGuo (d) or unsupplemented culture medium (e). (f) Day 14 thymus stained with F4/80 to reveal the distribution of Mϕ (the bar represents 100 μm).

recolonization of day 14 alymphoid thymi: in spite of abnormally high levels of class II expression following exposure to dGuo, the cortical epithelium failed to support positive selection of day 16 thymocytes. Interestingly, Kingston *et al.*[26] introduced single, micromanipulated stem cells into day 14 alymphoid rudiments and characterized the products of differentiation: while all other subsets were clearly represented, cells of a $CD4^+$ $CD8^-$ phenotype were only rarely detected, consistent with our findings.

In addition to expressing class II determinants for perusal

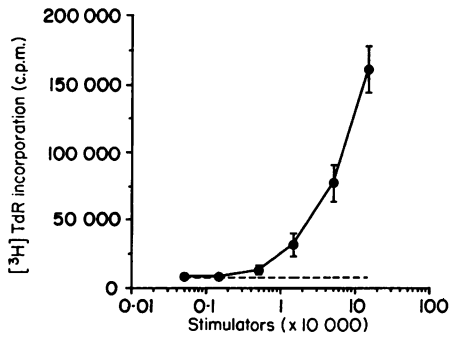

Figure 6. Response of purified C3H/He (H-2^k) T cells in an allogeneic MLR using freshly excised day 14 C57Bl/6 thymi as a source of stimulators. The broken line represents background counts.

by developing T cells, the thymic epithelium has been shown to secrete cytokines involved in the differentiation pathway.[27] Positive selection of mature T cells in day 14 fetal thymi could not be salvaged, however, by the addition of certain cytokines, expressed late during ontogeny. While rIL-2 and rGM-CSF actively retarded differentiation, IL-3 had no discernable effect on the CD4/CD8 profile: even rIL-1, shown previously to reverse the blockage in positive selection induced by mAb to class II,[28] failed to secure development of mature $CD4^+$ $CD8^-$ cells.

Although we cannot exclude the possible involvement of alternative cytokines, non-classical class II determinants such as I-O,[29] or unidentified co-stimulatory molecules expressed by the thymic epithelium,[25] it is perhaps significant that ultrastructural studies of the developing thymus from $(C57 \times DBA/2)F_1$ mice have shown that vascularization occurs at day 15 of ontogeny.[30] These findings suggest that blood-borne factors are excluded from the thymic primordium prior to this stage of development. Various experimental approaches have recently established a role for peptides in the positive selection of $CD4^-$ $CD8^+$ T cells.[31–33] Although the contribution of self-peptides to selection of class II-restricted thymocytes remains to be established, it is tempting to speculate that in the absence of vascularization, day 14 rudiments have yet to be exposed to those extrathymic self-proteins and related peptides that define the specificity of positive selection.

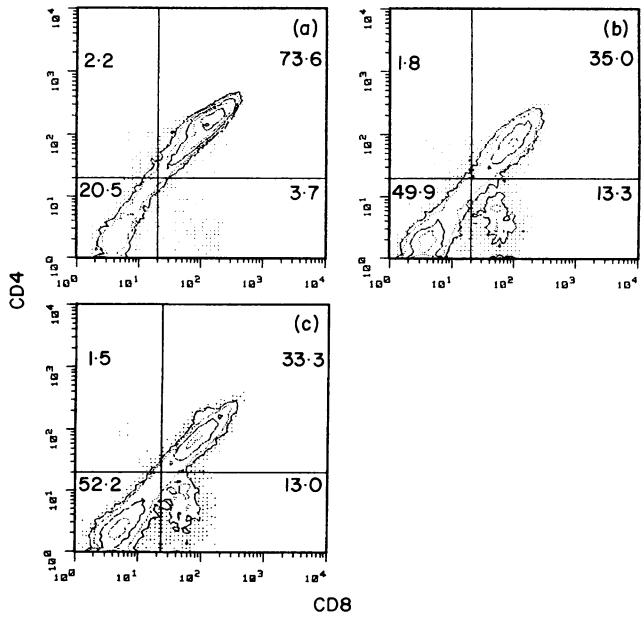

Figure 7. Effect of rGM-CSF and rIL-2 on thymocyte differentiation in day 14 rudiments. Thymi were cultured for 7 days in unsupplemented medium (a), or medium supplemented with either rGM-CSF (b) or rIL-2(c).

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