

TABLE 2 Nucleotide sequence homology in the use of Leu-Arg-Gly, Leu-Gly-Gly-Glu and Leu-Val-Ala-Gly

Sample		N-D-N-J		
KL3	AGCAGC	CTACGGGGGCCAAC	SS LRGAN	(V $\beta$ 5.2/J $\beta$ 2.6)
	AGCAGC	TTACGGGGACACCC	SS LRGTP	(V $\beta$ 5.2/J $\beta$ 1.2)
KL1	AGCAGC	TTGGCGCTGGCTAAT	SS LRLAN	(V $\beta$ 5.2/J $\beta$ 1.6)
	AGC	CAGTTGGCGCTTGGCTAAT	S Q LRLA	(V $\beta$ 5.2/J $\beta$ 1.6)
	AGCAGC	CAGTTGGCGCTTGGCTAAT	SS Q LRLA	(V $\beta$ 5.2/J $\beta$ 1.6)
	AGCAGC	TTGGATCGCTTGTATAAT	SS LDRLA	(V $\beta$ 5.2/J $\beta$ 1.6)
LJ1	AGC	ACGTTGGCGCTTGGGT	S T LRLG	(V $\beta$ 5.2/J $\beta$ 1.6)
	AGCAGC	CTACGGGGGCCAAC	SS LRGAN	(V $\beta$ 5.2/J $\beta$ 2.6)
	AGCAGC	CTACGGGGGCCAAC	SS LRGAN	(V $\beta$ 5.2/J $\beta$ 2.6)
MS18*	AGCAGC	TTGAGGGGGGCGCTA	SS LRGAL	(V $\beta$ 5.2/J $\beta$ 2.4)
BF1†	AGCAGC	CTCAGGGGG	SS LRG	(V $\beta$ 6/J $\beta$ 1.6)
E†	AGCAGC	ATAAGGGGAAGC	SS IRGS	(V $\beta$ 6/J $\beta$ 2.7)
BD3‡	AGCAGC	ATCGTCAGGGGATCG	SS IVRGS	(V $\beta$ 6/J $\beta$ 2.7)
ph 11‡	AGCAGT	TTAAGGGCGGGG	SS LRG	(V $\beta$ 8/J $\beta$ 1.1)
12H6§	AGCAGC	CTCGGGGACTTT	SS LRDF	(V $\beta$ 13/J $\beta$ 2.1)
KL3	AGCAGC	TTGGGAGGGTACCCTAT	SS LGGVPY	(V $\beta$ 5.2/J $\beta$ 1.2)
	AGCAGC	TTGGGAGGGTCCGAAGAG	SS LGGSEE	(V $\beta$ 5.2/J $\beta$ 2.3)
	AGCAGC	TTGGGAGGGTCCGAAGAG	SS LGGSEE	(V $\beta$ 5.2/J $\beta$ 2.5)
	AGCAGC	TTGGGAGGGTCCGTTGAG	SS LGGSV	(V $\beta$ 5.2/J $\beta$ 2.5)
4	AGCAGC	CTGGGGGGCGAA	SS LGGE	(V $\beta$ 8.2/J $\beta$ 2.5)
KL3	AGCAGC	TTAGTGGCGGGATCTATC	SS LVAGSI	(V $\beta$ 5.2/J $\beta$ 2.7)
	AGCAGC	TTAGTGGCGGGATCTATC	SS LVAGSI	(V $\beta$ 5.2/J $\beta$ 2.7)
	AGCAGC	TTGGTGGCGGGATCTATC	SS LVAGSI	(V $\beta$ 5.2/J $\beta$ 2.7)
	AGCAGC	TTAGTGGCGGGATCTATC	SS LVAGSI	(V $\beta$ 5.2/J $\beta$ 2.7)
KL1	AGCAGC	TTAGTGGCGGGATCTATC	SS LVAGSI	(V $\beta$ 5.2/J $\beta$ 2.7)
LJ1¶	AGCAGC	TTAGTGGCGGGATCTATC	SS LVAGSI	(V $\beta$ 5.2/J $\beta$ 2.7)
	AGCAGC	TTAGTGGCGGGATCTATC	SS LVAGSI	(V $\beta$ 5.2/J $\beta$ 2.7)
	AGCAGC	TTAGTGGCGGGATCTATC	SS LVAGSI	(V $\beta$ 5.2/J $\beta$ 2.7)
C†	AGCAGC	ATAGCTGGCGGT	SS IAGG	(V $\beta$ 6/J $\beta$ 2.3)

\* CDR3 usage in human MBP 88-99 specific T-cell line<sup>1</sup>.

† CDR3 usage in rat spinal cord derived T-cell clones specific for MPB 85-99 (ref. 2).

‡ Clone derived from a human tonsil cDNA library<sup>13</sup>.

§ Non-cytolytic mouse T-cell clone specific for influenza virus strain A/PR8/34 (ref. 15).

|| CDR3 usage in rat lymph node derived T-cell clone specific for MBP 85-99 (ref. 2).

¶ G → S substitution in J $\beta$ 2.7.

in bona fide clones with specificity for MBP 87-106 suggests strongly that T cells of such antigenic specificity are present in MS brain lesions, and that they represent a substantial fraction of the cells expressing V $\beta$ 5.2 TCR. An immune response directed against other antigens such as proteolipid protein, myelin-oligodendroglial glycoprotein and heat-shock proteins may also be critical in MS. As sequences for TCRs specific for these antigens become available, attribution of the specificity of some of the other rearrangements seen within lesions may be possible. □

Received 14 October; accepted 18 December 1992.

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ACKNOWLEDGEMENTS. We thank M. Davies for discussion. This work was funded by grants from the NIH, the National MS Societies of the United States and Australia, the Phil N. Allen Trust, the Rosenthal Fund and the National Health and Medical Research Council of Australia. M.A.P. is a Medical Student Research Training Fellow of the Howard Hughes Medical Institute.

## MHC class II-positive epithelium and mesenchyme cells are both required for T-cell development in the thymus

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T LYMPHOCYTES are produced in the thymus from precursors originating in the haemopoietic tissues. On entering the thymus, they undergo a programme of proliferation, T-cell receptor (TCR) gene rearrangement, differentiation and repertoire selection<sup>1</sup>. Although the thymus provides a unique environment for these events, the role of the thymic stroma in regulating specific developmental stages is not well understood<sup>2</sup>. We therefore devised an *in vitro* system to study the role of individual thymic stromal components in T-cell development. We report here that the development of TCR<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup> T-cell precursors into TCR<sup>+</sup> cells expressing CD4 and/or CD8 requires the presence of both major histocompatibility complex class II<sup>+</sup> epithelial cells and fetal mesenchyme. The requirement for mesenchymal support can be mapped to the initial stages of intrathymic development because the later stages of maturation, from double-positive CD4<sup>+</sup>CD8<sup>+</sup> thymocytes into single-positive CD4<sup>+</sup> or CD8<sup>+</sup> cells, can be supported by epithelial cells alone. We also show that the requirement for mesenchymal cells can be met by cells of the fibroblast line 3T3 (but not by supernatants from these cells). To our knowledge, these findings provide the first direct evidence that mesenchymal as well as

epithelial cells are involved in T-cell development, and suggest that their involvement is stage-specific and likely to be dependent on short-range or contact-mediated interactions.

TABLE 1 Thymic stromal cell requirements for T-cell development *in vitro*

Stromal cells	Thymocyte input	T-cell development in reaggregate cultures
Whole dGuO-treated stroma	14d TCR <sup>-</sup> CD4 <sup>-</sup> CD8 <sup>-</sup>	2/2*
Whole dGuO-treated stroma depleted of class II <sup>+</sup> epithelium	14d TCR <sup>-</sup> CD4 <sup>-</sup> CD8 <sup>-</sup>	0/3†
Purified class II <sup>+</sup> epithelium	14d TCR <sup>-</sup> CD4 <sup>-</sup> CD8 <sup>-</sup>	0/5‡
Purified class II <sup>+</sup> epithelium and fetal mesenchyme	14d TCR <sup>-</sup> CD4 <sup>-</sup> CD8 <sup>-</sup>	3/3*
Purified class II <sup>+</sup> epithelium and 3T3 Fibroblasts	14d TCR <sup>-</sup> CD4 <sup>-</sup> CD8 <sup>-</sup>	2/2*
Purified class II <sup>+</sup> epithelium	TCR <sup>-</sup> CD4 <sup>+</sup> CD8 <sup>+</sup>	3/3‡
Whole dGuO-treated stroma depleted of class II <sup>+</sup> epithelium	TCR <sup>-</sup> CD4 <sup>+</sup> CD8 <sup>+</sup>	0/2‡

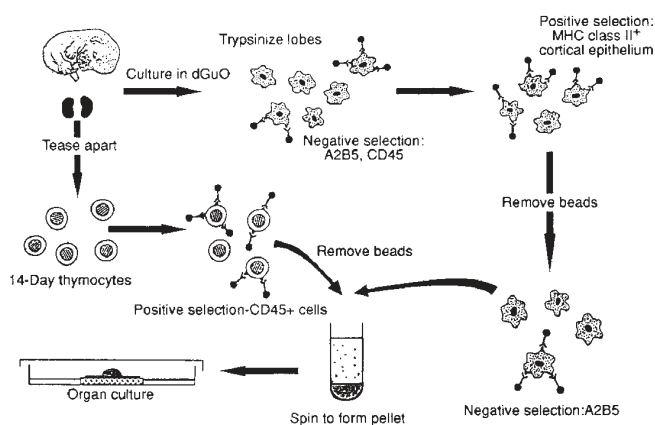
After 9 days, individual reaggregate cultures were gently teased apart to liberate any lymphoid cells. T-cell development was assessed on lymphoid content, and by CD4, CD8 and TCR expression, determined by three-colour flow cytometry (see legend to Fig. 3 for details). dGuO, deoxyguanosine.

\* Cell yields in successful reaggregate cultures showed a 2-5-fold increase over the lymphoid cell number added at the start of the culture. This probably represents an underestimate of the proliferative capacity of 14-day precursors because it is likely that not all cells are successfully incorporated into the reaggregates.

† Cultures recorded as unsuccessful contained less than 10% of the original input number.

‡ Cell yields from 4-day cultures of CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>-</sup> cells and purified epithelium were 20-30% of the input number, reflecting the limited life span of these cells unless rescued by positive selection<sup>14</sup>.

**FIG. 1** Techniques for preparing reaggregate organ cultures from selected thymocyte and thymic stromal cell populations. Thymic stromal cells were prepared by disaggregating deoxyguanosine (dGuO)-treated BALB/c fetal thymus lobes using 0.25% trypsin, 0.02% EDTA in  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free PBS. Residual cells of haemopoietic origin were removed from the suspension by three rounds of depletion using anti-CD45 (clone M1-9, ATCC)-coated anti-rat immunoglobulin magnetic beads (Dyna). The ability of anti-CD45-coated beads to bind to  $\text{CD45}^{+}$  cells is not prevented by prior trypsinization (our unpublished observations). To promote interaction, cells and beads were spun together in round-bottomed freezing vials (Sterilin) as described previously<sup>7</sup>. Stromal preparations lacking MHC class II<sup>+</sup> epithelial cells were obtained by immunomagnetically depleting suspensions using three rounds of anti-IA $\alpha$  (clone MK-D6, Becton-Dickinson) coated onto anti-mouse immunoglobulin beads. To prepare purified class II<sup>+</sup> epithelial cells, suspensions of CD45-depleted dGuO stroma were first immunomagnetically depleted of cells expressing the medullary marker A2B5 using anti-rat immunoglobulin beads coated sequentially with rat anti-mouse IgM (Pharmingen) and A2B5 hybridoma supernatant (a gift from M. Raff). Successful rosetting of A2B5<sup>+</sup> cells with antibody-coated beads was monitored by microscopy, confirming that A2B5 (a GQ ganglioside<sup>15</sup>) is protease-resistant. To obtain maximum depletion, beads and cells were spun together at a ratio of 5:1 in two successive rounds. Within each round, cells and beads were spun and resuspended twice. Rosetted cells and free beads were removed on a magnet after each round. MHC class II<sup>+</sup> epithelial cells were then positively selected from the remaining suspension using anti-IA $\alpha$ -coated beads at a bead:cell ratio of 2:1. Rosetted cells were collected on a magnet and washed four times to remove any unbound cells. After a final wash in PBS, rosettes were resuspended in 200  $\mu\text{l}$  pronase (10 mg ml<sup>-1</sup> in PBS; Sigma) at 37 °C for 2 min to remove the beads from the cells. The reaction was stopped by adding 800  $\mu\text{l}$  ice-cold medium containing 10% fetal calf serum (FCS) and the released beads plus any residual rosettes removed on a magnet. Free cells were collected by centrifugation and subjected to a further depletion with A2B5-coated beads as above. Reanalysis by flow cytometry showed these preparations to be free of A2B5<sup>+</sup> cells (data not shown). Fetal mesenchyme cells were prepared from the capsule of the fetal lung rudiment to avoid the possibility of contamination with non-mesenchymal thymic stroma. Isolated 14-day rudiments were incubated in 0.25% trypsin, 0.02% EDTA for 5 min, or until the outer mesenchymal



capsule was just beginning to dissociate. Gentle pipetting then released these cells leaving the remainder of the rudiment largely intact. 3T3 cells were grown as monolayers in flasks, collected by trypsinization and allowed to stand at 4 °C for 2 h before incorporation into reaggregates.  $\text{TCR}^{-}\text{CD4}^{-}\text{CD8}^{-}$  T-cell precursors were obtained by gently teasing apart freshly isolated 14-day fetal thymus lobes. The resultant suspensions were then positively selected using anti-CD45-coated beads as described above, except that beads were removed from rosetted cells using Detachabead (Dyna) according to manufacturer's instructions. This selection removes the possibility of contamination by fixed thymic stromal elements, although some cells or precursors of other haemopoietic lineage may be included in the selected population. Reaggregates were formed by mixing together the desired stromal and lymphoid components (usually at a ratio of 1:2 for epithelium and lymphocytes, and 2:4:1 for epithelium, lymphocytes and fibroblasts) and pelleting by centrifugation. After removal of the supernatant, the pellet was dispersed into a slurry, drawn into a fine glass pipette and placed as a standing drop on the surface of a nucleopore filter in organ culture. Within 12 h, intact thymic lobes reform from these mixtures (see Fig. 2).

Previous *in vitro* attempts to investigate the role of thymic stromal cells in T-cell development have been hampered by the limited ability of monolayer cultures to support a full programme of T-cell maturation and by the difficulty of obtaining cultures of thymic epithelial cells that maintain their *in vivo* phenotype<sup>3,4</sup>. We devised a technique (Fig. 1) to reaggregate defined thymocyte and stromal cell populations under organ culture conditions that provide optimal support for T-cell development *in vitro*<sup>5</sup>. Stromal cells were prepared by immunomagnetic selection from disaggregated 14-day fetal thymus lobes rendered free of lymphoid and major histocompatibility complex (MHC) class II<sup>+</sup> dendritic cells by culture in deoxyguanosine<sup>6</sup>. These lobes, after depletion of remaining  $\text{CD45}^{+}$  haemopoietic elements, provide a source of MHC class II<sup>+</sup> cortical epithelial cells, medullary epithelial cells and mesenchymal components<sup>7</sup>. Day-14 fetal thymocytes ( $\text{CD45}^{+}$ ,  $\alpha\beta$  TCR<sup>-</sup>, >97%  $\text{CD4}^{+}\text{CD8}^{-}$ ) were used as a source of T-cell precursors, and purified  $\text{CD4}^{+}\text{CD8}^{+}\text{TCR}^{-}$  cells provided an intermediate stage of thymocyte development. Mixtures of thymocytes and stromal cells rapidly reform (within 12 hours) into intact thymus lobes and were analysed for T-cell development in terms of lymphoid content and TCR, CD4 and CD8 expression after 9 days in culture (Fig. 2).

The results of associating various combinations of thymocytes and stromal cells are summarized in Table 1. In contrast to reaggregates using whole stromal cell preparations, lymphocytes were not recovered from reaggregate cultures of 14-day precursors and stromal preparations previously depleted of class II<sup>+</sup> epithelial cells. Purified class II<sup>+</sup> epithelial cells also failed to support precursor development, even though the epithelial cells reaggregated successfully (Fig. 2a, left) and retained a cortical epithelial phenotype including MHC class II expression (Fig. 2b). Such lobes remained small and were alymphoid on

collection. But when fetal mesenchyme was also incorporated, the reaggregates increased in size (Fig. 2a, right) and lymphoid content, and supported the development of  $\alpha\beta$  TCR<sup>+</sup> T cells expressing CD4 and CD8 (Fig. 3a, b).

Taken together, these results provide direct evidence that MHC class II<sup>+</sup> epithelial cells are necessary but not sufficient for T-cell development and show that, in addition to any non-lymphoid cells in the  $\text{CD45}^{+}$  input, mesenchymal elements are also required. But when double-positive  $\text{CD4}^{+}\text{CD8}^{+}\text{TCR}^{-}$ , rather than  $\text{CD4}^{+}\text{CD8}^{-}\text{TCR}^{-}$  cells were reaggregated with purified epithelium, further development did occur, resulting in the appearance of double-positive TCR<sup>+</sup> and single-positive  $\text{CD4}^{+}$  and  $\text{CD8}^{+}$  cells with upregulated levels of TCR expression (Fig. 3e-h). This pattern of differentiation *in vitro* parallels that seen as a result of TCR-mediated positive selection *in vivo*, and is accompanied by the development of functionally competent cells<sup>7</sup>. Thus the requirement for mesenchyme seems to be associated with the early stages of thymopoiesis, whereas epithelial cells alone can support the later developmental stages, including those dependent on TCR-mediated interactions with MHC antigens in the thymic stroma. In addition, the failure of class II-depleted thymic stromal cells to support the development of  $\text{CD4}^{+}\text{CD8}^{+}\text{TCR}^{-}$  cells (Table 1) indicates that class II<sup>+</sup> epithelial cells are essential, as well as being sufficient for these later stages of thymocyte maturation.

Because fetal mesenchyme is likely to consist of a heterogeneous mixture of cells, we investigated the mesenchymal components required for the initial stages of thymopoiesis. Therefore we examined the ability of 3T3 cells (a murine fibroblast cell line) to substitute for fetal mesenchyme. A combination of purified class II<sup>+</sup> epithelial cells and 3T3 cells (Fig. 3c, d) was able to support the maturation of 14-day precursors, but supernatants from confluent 3T3 cultures were not. These

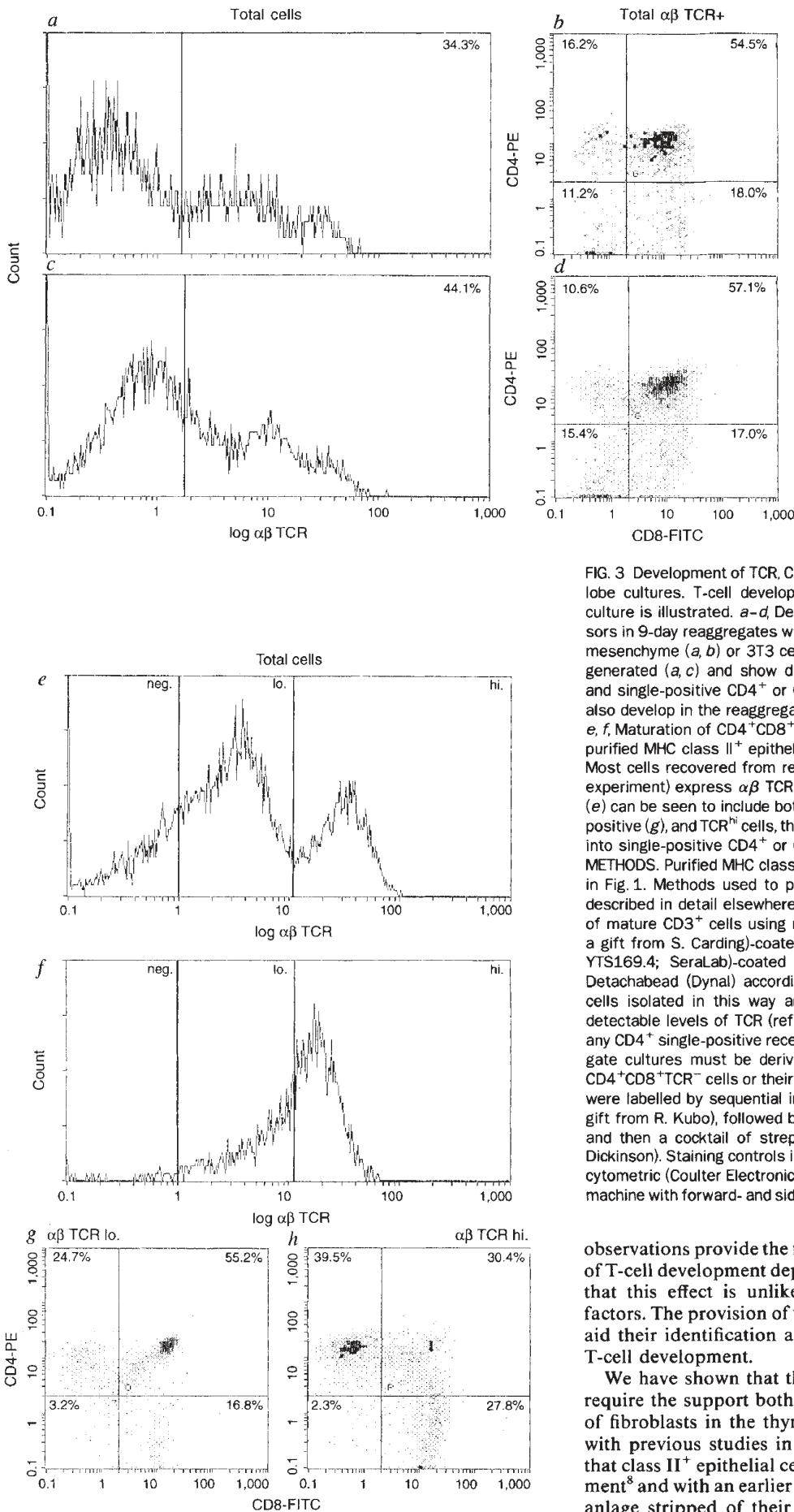


FIG. 3 Development of TCR, CD4 and CD8 expression in reaggregate thymus lobe cultures. T-cell development in two different types of reaggregate culture is illustrated. *a-d*, Development of CD4<sup>+</sup>CD8<sup>-</sup>TCR<sup>-</sup> 14-day precursors in 9-day reagggregates with purified class II<sup>+</sup> epithelium and either fetal mesenchyme (*a, b*) or 3T3 cells (*c, d*). In both cases, TCR<sup>+</sup> cells have been generated (*a, c*) and show differentiation into double-positive CD4<sup>+</sup>CD8<sup>+</sup> and single-positive CD4<sup>+</sup> or CD8<sup>+</sup> cells (*b, d*). (Note,  $\gamma\delta$  TCR<sup>+</sup> thymocytes also develop in the reaggregate cultures described above; data not shown.) *e, f*, Maturation of CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>-</sup> cells in 4-day reaggregate cultures with purified MHC class II<sup>+</sup> epithelium as the only type of stromal cell present. Most cells recovered from reagggregates (25% of the input number in this experiment) express  $\alpha\beta$  TCR (*f*) and by comparison with adult thymocytes (*e*) can be seen to include both TCR<sup>lo</sup> cells which are predominantly double-positive (*g*), and TCR<sup>hi</sup> cells, the majority of which have undergone maturation into single-positive CD4<sup>+</sup> or CD8<sup>+</sup> cells (*h*).

**METHODS.** Purified MHC class II<sup>+</sup> epithelial cells were isolated as described in Fig. 1. Methods used to purify CD4<sup>+</sup>CD8<sup>-</sup>TCR<sup>-</sup> thymocytes have been described in detail elsewhere<sup>7</sup>. Briefly, newborn thymocytes were depleted of mature CD3<sup>+</sup> cells using multiple rounds of anti-CD3 (clone C363.29B; a gift from S. Carding)-coated beads, then selected using anti-CD8 (clone YTS1.69.4; SeraLab)-coated beads. Bead removal was achieved using Detachabead (Dyna) according to the manufacturer's instructions. TCR<sup>-</sup> cells isolated in this way are >99.7% CD4<sup>+</sup>CD8<sup>+</sup> and do not express detectable levels of TCR (ref. 7, and data not shown). Using this approach, any CD4<sup>+</sup> single-positive receptor-bearing cells generated in these reaggregate cultures must be derived from precursors expressing CD8, that is, CD4<sup>+</sup>CD8<sup>-</sup>TCR<sup>-</sup> cells or their CD4<sup>-</sup>CD8<sup>+</sup>TCR<sup>-</sup> precursors. Cell suspensions were labelled by sequential incubations in anti- $\alpha\beta$  TCR (clone H.57-597; a gift from R. Kubo), followed by anti-hamster immunoglobulin biotin (Caltag) and then a cocktail of streptavidin APC, CD4-PE and CD8-FITC (Becton-Dickinson). Staining controls involved omission of first-step antibodies. Flow cytometric (Coulter Electronics, FL) analysis was done on an Elite Dual laser machine with forward- and side-scatter gates set to exclude non-viable cells.

observations provide the first direct evidence that the early stages of T-cell development depend on fibroblast products and suggest that this effect is unlikely to be mediated solely by soluble factors. The provision of these requirements by a cell line should aid their identification and analysis of their functional role in T-cell development.

We have shown that the initial stages of T-cell development require the support both of MHC class II<sup>+</sup> epithelial cells and of fibroblasts in the thymic stroma. Our results are consistent with previous studies in T-cell-deficient nude mice suggesting that class II<sup>+</sup> epithelial cells play a crucial role in T-cell development<sup>8</sup> and with an earlier report showing that fetal day-12 thymic anlage stripped of their mesenchymal capsule fail to support



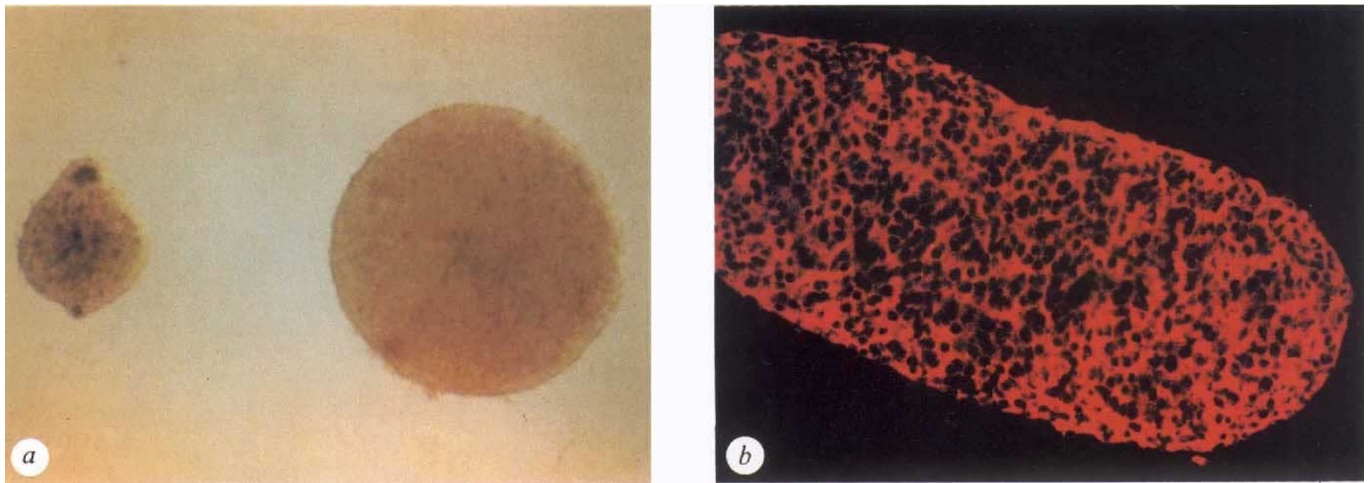


FIG. 2 Reaggregate cultures only support the development of 14-day CD4<sup>-</sup>CD8<sup>-</sup>TCR<sup>-</sup> thymic precursors when both epithelial and mesenchymal cells are present. *a*, Two age-matched reaggregate cultures, prepared as in Fig. 1. The lobe on the right was prepared by reaggregating 14-day thymocytes, purified class II<sup>+</sup> epithelial cells and fetal mesenchyme cells, whereas the other was prepared using 14-day thymocytes and purified epithelial cells alone. During the 9-day culture period, the lobe receiving mesenchyme cells increased in size and when collected showed a fourfold increase over the number of precursors added at the initiation of culture. Cells collected from such lobes show evidence of T-cell maturation comparable to that seen in unmanipulated cultures (Fig. 3). In contrast, the culture of precursors and purified epithelium alone remained small and was alymphoid, even though the epithelial cells had successfully formed an intact

structure and still expressed a cortical epithelial cell phenotype, including MHC class II antigen expression (*b*) and ERTR4 (ref. 16) and 4F11E (ref. 17) expression (not shown). In addition, such lobes were found to be negative for the macrophage markers 5C6 and F4/80 (ref. 18) and the reticular fibroblast marker ERTR7 (ref. 16).

**METHODS.** Reaggregate cultures were prepared as described for Fig. 1. For immunohistological analysis, cultures were embedded in OCT compound (Raymond Lamb, London) and snap-frozen in liquid nitrogen. Frozen sections were incubated sequentially in anti-mouse I-Ad (Becton-Dickinson) and alkaline phosphatase-conjugated anti-mouse immunoglobulin (Dako) with intervening washes. Slides were developed using a Fast Red TR substrate (Sigma) and viewed under fluorescence conditions as described previously<sup>19</sup>.

lymphocyte development<sup>9</sup>. They may also partly explain the limited success of attempts to induce a full programme of T-cell development using either monolayer cultures of a single thymic stromal cell type<sup>10</sup>, or cocktails of cytokines<sup>11,12</sup>. Having identified two key cell types required for T-cell development, our studies also provide a basis for an analysis of the cellular products regulating specific developmental stages. Epithelial cells and fibroblasts differ in their pattern of cytokine gene expression<sup>20</sup>, but it seems likely that fibroblasts may also influence T-cell maturation by contact mediated mechanisms. This might involve a direct effect on T-cell precursors, perhaps reflecting a continuation of the dependence of stem cells in the bone marrow on contact with fibroblasts or their matrix products<sup>13</sup>. Alternatively, indirect effects involving the regulation of epithelial cell function by mesenchyme are also possible. □

## Decoration of the microtubule surface by one kinesin head per tubulin heterodimer

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**KINESIN**, a microtubule-dependent ATPase, is believed to be involved in anterograde axonal transport. The kinesin head, which contains both microtubule and ATP binding sites, has the necessary components for the generation of force and motility<sup>1</sup>. We have used saturation binding and electron microscopy to examine the interaction of the kinesin motor domain with the microtubule surface and found that binding saturated at one kinesin head per tubulin heterodimer. Both negative staining and cryo-electron microscopy revealed a regular pattern of kinesin bound to the microtubule surface, with an axial repeat of 8 nm. Optical diffraction analysis of decorated microtubules showed a strong layer-line at this spacing, confirming that one kinesin head binds per tubulin heterodimer. The addition of Mg-ATP to the microtubule-kinesin complex resulted in the complete dissociation of kinesin from the microtubule surface.

The kinesin ATPase has a low rate of ATP hydrolysis which is stimulated ~1,000-fold by microtubules<sup>2-5</sup>. The precise

Received 8 October; accepted 29 December 1992.

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**ACKNOWLEDGEMENTS.** We thank A. Murdoch and D. McLoughlin for technical support and A. Orchard for help with photography. The work was supported by an MRC programme grant to E.J.J., G.W. and J.J.T.O. G.A. is the holder of a Wellcome Prize Studentship.

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