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Interleukin-7: A Cofactor for V(D)J Rearrangement of the T Cell Receptor β Gene

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The diversity of the T cell receptor repertoire is generated by rearrangement of gene elements in immature thymocytes. To identify a thymic signal that induces this rearrangement, a variety of agents were tested for their ability to induce rearrangement of the T cell receptor β gene in suspensions of thymocytes from mouse embryos at day 14 of gestation. Of 16 agents tested, only interleukin-7 (IL-7) induced V(D)J gene rearrangement and sustained expression of the *RAG-1* and *RAG-2* genes, which are known to control rearrangement. These data implicate IL-7, a cytokine that is abundantly expressed in embryonic thymus, in driving gene rearrangement during early T cell development.

The antigen receptors of lymphocytes are encoded by genes that are assembled from separate gene elements (*1*). During rearrangement of the T cell receptor β (TCRβ) gene (*2*, *3*), variable (V), diversity (D), and joining (J) elements are cut, recombined, and joined together (*4*, *5*), creating the enormous diversity of T cell receptors. Similar phenomena occur in the TCRα, γ, and δ genes in T cells and in immunoglobulin (Ig) genes in B lymphocytes. This gene rearrangement appears to be strictly regulated: only precursor lymphocytes rearrange and the process occurs in a certain sequence. Thymocytes for example, first rearrange the D to J elements, then the V to D elements in the TCRβ locus, and later the TCRα locus is rearranged. We investigated the signals from the thymic microenvironment that induce rearrangement of the TCRβ locus.

Because the precursor T cells (pre-T cells) in which the rearrangement occurs are few in number, we used a polymerase chain reaction (PCR) for detection of V_β rearrangement (Fig. 1A). V and J regions are separated by 200 to 600 kb in the germline configuration (too long a span to generate a PCR product). If these regions are joined by the rearrangement process in a T cell, the PCR amplification product is 330 bp; the specificity of this product was verified by hybridization with a probe located between the primers. We used this technique to detect rearrangement of $V_\beta 8$ (Figs. 1 and 2), $V_\beta 3$, $V_\beta 6$, and $V_\beta 11$ (*6*).

To find an unrearranged T cell precursor population, we analyzed thymic subsets from adult mice. Triple negative cells ($CD4^-CD8^-CD3^-$), which represent 2% of the adult thymus, do not yet express TCR proteins. Nonetheless, most had already rearranged the TCRβ locus and were therefore not useful for these studies (*7*). We next examined fetal hematopoietic tissue to define the onset of V_β rearrangement (Fig. 1B). T cell precursors are generated in the fetal liver; they then migrate to the thymus where we detected V(D)J rearrangement at day 15 of gestation (longer autoradiographic exposure revealed presumably postthymic cells in the liver at day 19). A similar time frame for V_β rearrangement had previously been shown with the use of hybridomas generated from thymocytes at different time points during fetal development (*8*). At day 14, we detected less than one rearranged cell per thymus lobe (containing approximately 30,000 cells).

We therefore used these pre-T cells to study signals for rearrangement. The intact thymus was cultured, and the timing of V(D)J rearrangement was assayed by PCR (Fig. 2). V(D)J rearrangement occurred between 24 and 48 hours ($V_\beta 8$ is shown in Fig. 2, but the result was also reproduced for $V_\beta 3$ and $V_\beta 6$). For quantitation of the rearranged DNA, titration (*9*) indicated at least a 500-fold increase during 48 hours of organ culture, far more than could be a result of cell proliferation. The time course of rearrangement in organ culture resembled that seen in vivo (Figs. 1B and 2A). Thus, the isolated thymus provided a sufficient environment for rearrangement. Fetal thymic organ culture has required floating the organ on medium to achieve growth and differentiation processes (*10*); however, we observed no difference in the rates of V(D)J rearrangement within the first 48 hours in floating versus immersion cultures of the organ (*6*).

Intact organ structure was required for V(D)J rearrangement in vitro (Fig. 2B) because no rearrangement was detectable if the thymocytes were cultured as suspensions. Even when thymocyte suspensions were cultured in U-bottom plates (in order to provide more cell contact among the

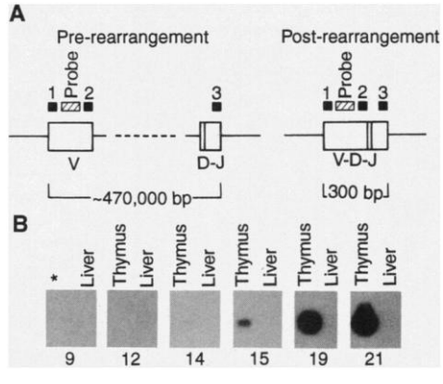

Fig. 1. V(D)J rearrangement of the TCRβ gene during fetal development. (**A**) Diagram of the murine TCRβ gene before (left) and after (right) rearrangement. Locations are indicated for PCR primers 1, 2, and 3 (below), as well as for the probe used for detecting the PCR product by hybridization (Probe). (**B**) PCR with DNA from C57Bl/6 embryonic mice. Organs were removed on the indicated days, and DNA was extracted (*26*) and quantitated. A constant amount of DNA (1 μg) from each treatment group was analyzed by PCR with the $V_\beta 8.1,2,3$ primer (primer 1, GAGGCTGCAGTCACCCAAAGTCCAA) and with opposite strand primers for $V_\beta 8.1,2,3$ (primer 2, ACAGAAATATACAGCTGTCTGAGAA) or $J_\beta 2.1$ (primer 3, TGAGTCGTGTTCCTGGTCCGAAGAA). The reaction mixture contained 35 mM tris (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, bovine serum albumin (100 μg/ml), nucleoside triphosphates (NTPs) (2 μM each), the primers (0.5 μM each), and 1 U of Taq polymerase. The amplification was performed for 30 cycles (1 min at 94°C, 2.5 min at 54°C, and 1.5 min at 70°C, with a 7-min extension period). The PCR product was then separated on a 1.2% agarose gel and blotted. The specific product of 330 bp was detected by hybridization with a ^{32}P-labeled oligonucleotide probe (100 mer) from the $V_\beta 8.1$ sequence between primers 1 and 2. Prehybridization and hybridization were performed at 54°C overnight in 6× sodium saline citrate (SSC), 5× Denhardt's solution, and denatured salmon sperm DNA (50 μg/ml). Membranes were washed 20 min at room temperature and then for 20 min at 54°C in 6× SSC. The membranes were then exposed for autoradiography overnight. This PCR method is relatively quantitative, in that the amount of product is related to the amount of rearranged DNA, and increasing the number of cycles gives more product (*9*). This experiment, showing the onset of rearrangement in the thymus at day 15, is representative of three separate experiments. Asterisk indicates embryo without liver.

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single cells), rearrangement was not observed (6). This suggested that thymocytes required continuous signals from the thymic microenvironment to rearrange the V_β genes. These signals were apparently present in the intact organ but not in the cell suspension cultures.

To identify the factors provided by intact thymus, we screened a variety of agents known to activate immature or mature T cells for the ability to induce rearrangement in thymocyte suspension cultures (Table 1). Only interleukin-7 (IL-7) restored V(D)J rearrangement in fetal thymocyte suspension culture (Fig. 2C). IL-7 promoted rearrangement in fetal thymocytes but not in fetal liver cells (Fig. 2C), which indicates that it has a selective effect.

Comparison of cell recovery after culture showed IL-7 had an intermediate effect compared to other treatments [phorbol myristate acetate (PMA) + ionomycin > stem cell factor (SCF) > IL-7 > IL-1 > IL-2 > medium] (Figs. 2 and 3). Phenotypic analysis by flow cytometry of untreated cells and cells cultured 2 days in IL-7, SCF, or PMA and ionomycin showed the presence of about 88% Thy-1^+ cells (86, 89.4, 88.1, and 89.4%, respectively) and an average of 65% Pgp-1^+ cells (43.8, 62.6, 82.3, and 69.3%, respectively). Thus, all of these treatments maintained precursor T cell viability, but only IL-7 induced rearrangement.

IL-7 mRNA is expressed in adult and fetal thymus (*11*, *12*) (Fig. 3), and its source appears to be epithelial cells (*13*). IL-7 mRNA was not found after the suspension culture (Fig. 3), possibly because thymic epithelial cells are disrupted or require cell contact for IL-7 production. Thus, in these cell suspensions, the limiting factor for TCRβ rearrangement appears to be IL-7.

How does IL-7 promote V(D)J rearrangement? The nuclear machinery for rearrangement has not been defined yet, but two gene products, RAG-1 and RAG-2, induce rearrangement upon transfection into fibroblasts (*14*, *15*). Both *RAG* genes are essential for rearrangement, which is completely abolished in mice by disruption of either gene (*16*, *17*). IL-7 treatment maintained *RAG-1* and *RAG-2* mRNA expression for 24 hours in our suspension culture (Fig. 3). Other stimuli (PMA and ionomycin, SCF, or IL-1) that failed to support rearrangement also failed to sustain *RAG* mRNA expression, although they maintained cell viability and mRNA for actin (Fig. 3). Thymocytes that were kept in suspension culture for 24 hours without any stimulus had no detectable *RAG* gene expression (6). Because the starting population of thymocytes at day 14 expresses the *RAG* genes, the role of IL-7 in this in vitro

Table 1. Stimuli tested for the ability to induce V(D)J rearrangement of the TCRβ gene in fetal thymocyte suspensions. Con A, concanavalin A; TNF, tumor necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; TGF-β, transforming growth factor–β; PHA, phytohemagglutinin; cAMP, cyclic adenosine monophosphate.

Agent	V(D)J rearrangement of TCRβ
IL-7	+
IL-7 + SCF	±
SCF	–
IL-1	–
IL-1 + Con A	–
TNF + IL-2	–
PMA + IL-4	–
PMA + ionomycin	–
PMA	–
Ionomycin	–
IL-2	–
GM-CSF	–
TGF-β	–
Con A	–
PHA	–
Dibutyryl cAMP	–
Supernatants of	
Con A + spleen cells	–
Embryonic thymus	–
Adult thymus	–

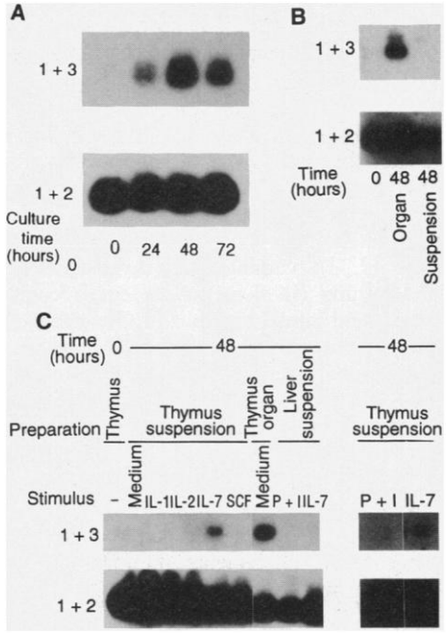

Fig. 2. IL-7 promotes V(D)J rearrangement of the TCRβ gene in fetal thymocytes. (**A**) A time course of rearrangement in vitro was observed in over 30 experiments. (**B**) A similar failure to rearrange in cell suspensions was observed in over 20 experiments. (**C**) Similar IL-7 induction of rearrangement was observed in ten experiments. Fetal thymus was removed at day 14 of gestation and placed in a culture well (96-well plates; one thymus lobe per well) in 50 μl of RPMI-1640 medium supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), 10% fetal calf serum, and 2-mercaptoethanol (5×10^{-5} M). DNA was extracted after the indicated times and quantitated, and 1 μg was analyzed by PCR as described (Fig. 1). For (B) and (C), we produced thymus suspensions by disrupting thymuses with repeated passage through a micropipette tip. Fetal liver suspensions were produced by repeated passage through a 3-ml syringe. The concentrations of agents added to the culture in (C) were 10 ng/ml (IL-1, IL-2, and IL-7), 100 ng/ml (SCF), 20 ng/ml [PMA (P)], and 0.5 μg/ml [ionomycin (I)]. After culture, DNA was extracted and quantitated as described (Fig. 1), and 1 μg was analyzed by PCR. To verify equal loading, we also analyzed samples by using primer sets 1 and 2 that react with either unrearranged or rearranged $V_\beta 8$. The recovery of cells after 48 hours of treatment in medium alone, IL-1, IL-2, IL-7, SCF, or P + I was 5.8 ± 3.6, 53.5 ± 21.1, 28.0 ± 12.4, 82.0 ± 13.9, 132.3 ± 27.6, and 284.3 ± 35.2, respectively (mean ± SD from four separate experiments). The primers used are indicated on the left of each panel.

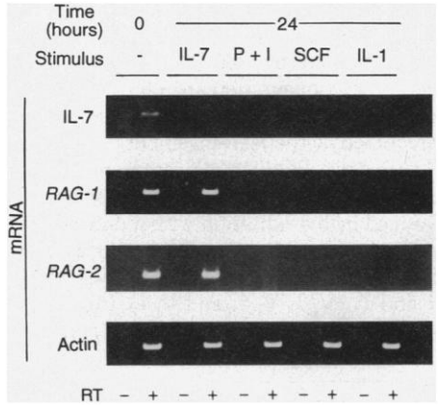

Fig. 3. IL-7 promotes *RAG* gene expression in thymocyte suspension cultures. Thymocyte cell suspensions were produced and cultured 24 hours with the indicated agents as in Fig. 2. RNA was extracted with the use of RNA STAT-60, reverse-transcribed with 1 μg of oligo(dT), NTPs (2 mM each), 5 mM dithiothreitol, and 1 U of murine Moloney leukemia virus reverse transcriptase (RT) in a total buffer volume of 20 μl [buffer was 50 mM tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$]. Complementary DNA was produced from equal numbers of cells placed in culture. PCR amplification was performed as described (Fig. 1) with the use of primers for IL-7, actin (*12*), *RAG-1*, and *RAG-2* (*27*), and the product was stained with ethidium bromide. Reverse transcriptase was omitted in alternate lanes as a control. The experiment shown, in which IL-7 sustained expression of *RAG-1* and *RAG-2*, is representative of four independent experiments. The recovery of cells after 24 hours of treatment in medium alone, IL-1, IL-2, IL-7, SCF, or P + I was 14.3 ± 8.5, 23.0 ± 3.3, 27.8 ± 10.7, 110.5 ± 28.9, 91.3 ± 12.1, and 165.5 ± 24.5, respectively (mean ± SD from four separate experiments).

system appears to be in maintaining *RAG* expression, which then supports V(D)J rearrangement. We cannot determine whether the *RAG* gene expression in the starting population was a response to IL-7 previously encountered in vivo, which would be possible because the IL-7 and *RAG* genes are all first detected at about the same time (day 12) of embryogenesis.

IL-7 (*11*) serves as a growth factor rather than as an inducer of Ig gene rearrangement in early B cell development (*18*). Large amounts of IL-7 mRNA in the thymus implicate IL-7 in T cell development also (*12*) (Fig. 3). IL-7 is required for thymocyte development (*19*) and can sustain the viability of immature thymocytes in suspension cultures without inducing cell proliferation (*20, 21*). Transgenic mice that overexpress IL-7 display more of all subsets of thymocytes (*22*). We assume that the IL-7 effects in these and our studies are due to direct effects on thymocytes, which display IL-7 receptors (*23*), although indirect effects by means of other cells cannot be excluded.

Our data suggest that IL-7 is an essential cofactor for V_β rearrangement in precursor T cells. In addition to sustaining *RAG* expression, IL-7 could also promote rearrangement by other means. IL-7 could enhance the production or activity of components of a recombinase complex (*24*). Considering that transcription has also been suggested to be necessary for rearrangement (*25*), IL-7 could promote transcription of the V_β genes.

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Chemorepulsion of Axons in the Developing Mammalian Central Nervous System

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During development of the nervous system, distinct populations of nerve cells extend specialized processes, axons and dendrites, over considerable distances to locate their targets. There is strong evidence for two general mechanisms by which these connections are made. The first involves attractive and repulsive interactions, both between cells and between them and their extracellular matrix. The second depends on the release of diffusible chemoattractants by target structures. Evidence is now provided for a mechanism of axon guidance in which diffusible chemorepulsive factors create exclusion zones for developing axons, causing them to turn away from inappropriate territory.

Axons reach their final destinations by locating successive intermediate targets (*1, 2*). Thus, it is important to understand the local control of the axon and its terminal sensory-motor apparatus, the growth cone. Interactions between growth cones and their environment may promote the advance of axons by contact guidance (*2*) or cause their arrest by contact inhibition (*3*). Guidance also occurs as a function of diffusion gradients, in which case both intermediate (*4*) and final targets (*5*) release diffusible factors that attract developing axons.

If guidance mechanisms that operate by cell-cell contact can be either attractive or repulsive, then attractive guidance mechanisms operating by diffusible factors might have counterparts in repulsion. I have examined this possibility in the olfactory system of the embryonic rat. Here, the olfactory bulb is continuous posteriorly with the anterior olfactory nucleus, which connects medially with the septum, laterally with the lateral olfactory cortex, and superiorly with the cerebral cortex. Around embryonic day 15 (E15) of development, mitral and tufted cells of the olfactory bulb are arranged concentrically. They project axons that turn within the bulb away from the midline and emerge laterally on the surface of the brain to form the lateral olfactory tract (LOT) (Fig. 1, a and b) (*6, 7*). At this stage, LOT axons form a discrete bundle immediately beneath the pial surface over the lateral part of the olfactory cortex. Only later, at E17 to E18, do these axons extend collateral branches that innervate the olfactory cortex, and at no stage do they innervate the cerebral cortex (*6*). It is possible that the divergence of these axons away from the midline is caused by the release of diffusible chemorepulsive factors from nearby medial structures.

To test this hypothesis, I cocultured explants of septum and olfactory bulb from E14.5 to E15 embryos 100 to 400 μm apart for 40 to 48 hours in collagen gel matrices, which establish gradients of diffusible factors (Fig. 2a) (*4, 5*). Under these conditions (*8*), axons from olfactory bulb explants consistently grew away from the septum, the outgrowth from which was unaffected ($n = 87$) (Fig. 2b). Explants of E14.5 to E15 lateral olfactory cortex ($n = 8$) or cerebral cortex ($n = 8$) also produced inhibitory effects on the growth of olfactory bulb axons. However, when cocultured with E18 olfactory cortex, E14.5 to E15 olfactory bulb explants grew radially and in equal numbers, so that 28.5 ± 2.13%, 22.5 ± 2.28%, and 49.8 ± 1.88% (mean ±

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