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ANALYSIS OF THE FIRST TWO WAVES OF THYMUS HOMING STEM CELLS AND THEIR T CELL PROGENY IN CHICK-QUAIL CHIMERAS

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The avian embryo and its accessibility in the egg offer significant advantages for the analysis of immune system development. The separate developmental pathways of thymus-dependent T cells and bursa-dependent B cells (1-4) and their derivation from hemopoietic stem cells (SC) (5-7) were first disclosed in studies of chick development. Chick-quail chimeras were used to show that blood-borne stem cells periodically migrate into the thymus in response to chemoattractants produced by thymic epithelial cells (8, 9). The first wave of stem cell influx into the thymus begins on the fifth day of embryonic development (E5) in the quail and on E6.5 in the chick, and this influx persists for 1-2 d. At least two additional waves of stem cells enter the embryonic thymus after nonreceptive intervals of ~4 d (9, 10).

The development of mAbs with specificity for the chicken TCRs and associated cell surface molecules (CD3, CD4, CD8) has allowed detailed study of the T cell progeny of hemopoietic stem cells (11-18). Sequential development of three sublines of daughter T cells has been defined using these antibodies. The first consists of T cells expressing the avian TCR-γ/δ homologue in association with the avian CD3 protein complex (13). These TCR-1/CD3-bearing cells appear first in the E12 thymus. T cells expressing the avian α/β receptor complex, TCR-2/CD3, appear on E15 (14-16). As in mammals, the immature TCR-2 cells express both CD4 and CD8 molecules, whereas the TCR-1 thymocytes express neither. A third T cell sublineage has recently been identified as cells expressing a different receptor complex (17, 18). These TCR-3/CD3+ cells begin to appear in the thymus on E18.

The present experiments, using chick-quail chimeras constructed by embryonic thymus engraftment, were primarily designed to examine lineage relationships among the different waves of stem cells and their intrathymic progeny. Specifically, we sought
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to determine whether each wave of stem cells entering the thymus gives rise to one or more of the T cell sublineages that express either TCR-1, TCR-2, or TCR-3. Second, we wished to examine the microanatomical features that characterize intrathymic differentiation of precursor T cells. A third objective was to examine the initial migration pattern of chick thymus-derived cells. This analysis was possible in chick-quail chimeras because the mAb markers of chick T cells do not react with quail T cells. The CT1 mAb, which identifies both quail and chick thymocytes (19), provided a useful exception to this rule.

Materials and Methods

Animals. Outbred quail (Coturnix coturnix japonica) and chick (Gallus gallus) embryos were used throughout this study. Fertile eggs, bought from commercial sources, were incubated at 40°C and the embryos staged according to the number of days of incubation.

Transplantations. Interspecies thymus grafts were used to study the TCR isotypes expressed by the progeny of waves. For the first wave, the chick thymic rudiment was taken from a 9-d embryo (E9) and grafted into an E3 quail. For the second wave, a 7-d quail thymic rudiment was grafted into an E3 chick. The grafts were implanted into the somatopleure according to a previously described method (10). The duration of the graft was 5-18 d.

Cell Suspensions. Thymic grafts were mechanically disrupted by gentle crushing between ground glass slides and washed in PBS containing 10% newborn calf serum.

Antibodies. The mAbs produced in BALB/c mice recognize the chicken CD3 molecular complex (CT3; reference 11), y and δ chain heterodimers of the TCR (TCR-1; reference 13), α and β chain heterodimers (TCR-2; references 14 and 15), the TCR-3 heterodimer (18), CD4 (CT4; reference 12), CD8 (CT8; reference 12), and a common thymocyte antigen (CT3a; reference 19). All of these mAbs are reactive with chick but not with quail thymocytes. The exception to this rule is the CT1 mAb, which reacts with a common thymocyte antigen present in both chicks and quails (19).

Immunostaining. Viable cells (10⁶) were incubated with the specific mAb and, after washing, they were labeled in a second step with goat anti-mouse IgG-FITC (Southern Biotechnology Associates, Birmingham, AL). Immunofluorescence was analyzed by automated flow cytometry using a FACScan instrument (Becton Dickinson Immunocytometry Systems, Mountain View, CA). An immunoperoxidase method using the mouse mAbs, biotin-derivatized horse antibodies to mouse IgG (Vector Laboratories, Inc., Burlingame, CA), an avidin-biotin peroxidase complex reagent (Vector Laboratories, Inc.), and the substrate 3,3′ diaminobenzidine (Sigma Chemical Co., St. Louis, MO) was used as described to stain tissue sections (16).

Results

Population Kinetics of the Thymocyte Progeny of the First Wave of Stem Cells. The donor thymus for these experiments was derived from E9 chick embryos, i.e., 1 d after completion of the influx of the first stem cell wave (10). When placed in the quail embryo (E3), this wave of chick stem cells gave rise to all of the normal chick thymocyte subpopulations. The developmental patterns of the TCR-1 and TCR-2 subpopulations in the transplanted thymus are shown in Fig. 1. When these experiments were performed, the TCR-3 antibody was not available for analysis of the viable cells by surface staining. When stored tissue sections were examined later, TCR-3 cells were observed in the thymus transplants beginning on E21 and in all older specimens.

While the TCR-1, TCR-2, and TCR-3 subpopulations were all generated as the intrathymic progeny of the first wave of stem cells, this first growth of T cells was limited in duration (Fig. 1). Plateau levels of chick thymocytes were reached by the thymus donor age of E18. Their frequency then declined rapidly beginning at E22,
Embryonic Age of Transplant (in days)

Figure 1. Thymocyte progeny of the first stem cell wave. Surface immunofluorescence staining of cells recovered from chick thymus (E9) transplanted into an E3 quail. Note that the CT1a mAb is a universal marker for chick thymocytes. The CT4 and CT8 mAbs identify almost all of the non-TCR1+ thymocytes of chick origin.

so that very few chick thymocytes remained in the thymus graft by E27. By this time, the cortex of the chick thymus transplant was repopulated almost entirely by thymocytes of recipient quail origin. This population was identifiable by the CT1 antibody but not by the CT1a chick-specific antibody marker (Fig. 2). The replacement thymocyte population was therefore derived from the succeeding wave of quail stem cells.

Kinetics of the Thymocyte Progeny of the Second Wave of Stem Cells. The differentiation potential of the second wave of stem cells was analyzed by grafting an E7 quail thymus (i.e., 1 d after entry of the first wave of stem cells; reference 9) into an E3 chick. Both the TCR-1 and TCR-2 subpopulations were also generated from second wave stem cells, but a sequential developmental pattern was not obvious for these subpopulations (Fig. 3). These observations were confirmed in a second experiment that gave virtually identical results. The tissues from these experiments were not preserved for subsequent analysis of the TCR-3 subpopulation.

Microenvironmental Relationships of the Thymocyte Progeny of the First Stem Cell Wave. The TCR-1 cells, normally generated in the thymic cortex from E12 onward, begin to migrate into the medullary region by E13. In contrast, the cortico-medullary transit of the TCR-2 population is not appreciable until E17 (Bucy, R. P., C. H. Chen, and M. D. Cooper, manuscript in preparation). Since the chick thymus implants received quail stem cells in subsequent attractant phases, the chick-quail chimera
Figure 2. Immunoperoxidase staining of chick thymus transplant at E27 embryonic age (E9 chick thymus 18 d after transplantation to an E3 quail). (A) Stained with CT1, which recognizes both quail and chick thymocytes. (B) Stained with CT1a, which is specific for chick thymocytes. Methyl Green counterstain (original magnification, x400).
model provided an opportunity to observe the maturation and migration patterns for the thymocyte progeny of a single stem cell wave.

As expected from the cell surface immunofluorescence analysis (Fig. 1), immunohistological assessment revealed an entirely normal pattern of initial thymocyte development in the E9 thymus implants. However, the thymic cortex was depleted of TCR-1 cells by E19, when virtually all of the TCR-1 cells were located in the medulla (Fig. 4). At this time the thymocytes in the outer cortex were still predominantly CD3+, but over the next several days the subcapsular cortex was occupied by CD3−, CT1a−, CT1+ cells, indicating replacement of chick thymocytes by the thymocyte progeny of quail stem cells. In the E27 chick thymus, only a few TCR-2+ cells remained in the cortex (Fig. 5). Most of the residual chick T cells were located in the medulla of the transplanted chick thymus, which by this time was largely repopulated with quail thymocytes (Fig. 2).

Migration of Chick Thymocytes to Quail Peripheral Lymphoid Tissues. Chick T cells normally begin to migrate into the peripheral lymphoid tissues a few days before hatching (11). In the chick-quail chimeras, chick T cells were found in the spleen, bursa, and intestine beginning ~7-10 d after transplantation of the E9 thymus. The lowest frequency of peripheral T cells was observed in the intestine (Fig. 6). Peripheral seeding of TCR-1 cells was initiated essentially on the normal development schedule, whereas the subsequent seeding of TCR-2 cells was significantly delayed in the chimeric host (Fig. 7).

Because a population of unusual lymphoid cells, which express cytoplasmic CD3 determinants, can be detected in the chick spleen and bursa from E8 onward, we searched for cells with this phenotype in the peripheral lymphoid tissues of the chick-quail chimeras. However, CD3+ cells could not be found in peripheral lymphoid tissues of the chick thymus recipients before the appearance of TCR-1+ cells.

Migration of Chick Thymocytes to the Quail Thymus. Chick T cells of both TCR-1 and TCR-2 sublineages could be regularly observed in the quail thymus beginning ~8 d after thymus transplant. These immigrant T cells were confined to the medulla (Fig. 8).

Discussion

These results are of interest from several points of view. First, they demonstrate that all three T cell sublineages expressing the TCR-1, TCR-2, and TCR-3 isotypes
Figure 4. Continued on facing page.
Figure 4. Immunoperoxidase staining of chick thymus transplant at donor age of E19. Primary mAb: (A) CT3; (B) CT4; (C) CT8; (D) TCR-1; (E) See next page. TCR-2. Methyl Green counterstain (original magnification, ×200).
are derived from the first wave of stem cells (SC) that enter the embryonic thymus. This generalization appears to apply to the second wave of SC as well, at least for the TCR-1 and TCR-2 sublines, which were the only ones examined in these quail-chick chimeras. All three T cell sublines thus appear to be generated in a biorhythmic cascade by the entry of successive waves of blood-borne SC. It will be of considerable interest to determine whether the receptor repertoires expressed by the progeny of the different SC waves are the same or different. Although the pattern of SC influx into the thymus has not been so extensively analyzed in other species, evidence exists for similar waves of SC influx in the mouse (20) and in the frog (21). Moreover, the patterns of Vγ and Vδ utilization in mice differ as a function of developmental age (22–24).

The present analysis of the first wave of SC and the duration of the intrathymic sojourn of their thymocyte progeny conforms well with results of earlier studies using nuclear morphology to discriminate chick and quail cells in thymic chimeras (9, 10). From the time of initial SC influx in the E6.5 chick, it takes ~2 wk to reach the peak level of T cell generation in the thymus, and the duration of the first thymocyte wave is ~3 wk.

The rates of cortico-medullary migration by the TCR-1 and TCR-2 subpopulations are notably different. TCR-1 cells can apparently make the transit from cortex to medulla in 1 d (E12–E13) and the cortex is substantially depleted of the first TCR-1 population by E19. In contrast, the initial TCR-2 population does not begin to enter the medulla until E17 and remains relatively abundant in the thymic cortex.
on E23. This relatively rapid passage of the TCR-1 cells through the thymus may be an indication of a lack of repertoire selection for this subpopulation.

The peripheral seeding of the TCR-1 progeny of the first SC wave appeared to occur normally in the chick-quail chimeras, whereas seeding of the TCR-2 cells was delayed by \( \sim 5 \) d. The basis for this selective delay in migration from the thymus was not revealed by the present experiments. In parallel studies, in which embryonic spleen, bursa, and intestine were transplanted, TCR-1 and TCR-2 cells were not generated outside of the thymus (25).

The second wave of SC, examined in chick embryos (E3) receiving a quail thymus (E7) containing its own first wave of SC, also gave rise to both TCR-1 and TCR-2 cells. However, a sequential developmental pattern for the two subpopulations was not evident in experiments of this design. The SC in both the first and second wave studies were derived from young chick embryos, but the thymic microenvironment provided for the second wave of SC differed in that it was from an older quail embryo. These data suggest that the thymic microenvironment may govern the temporal sequence of TCR gene rearrangement and expression. Additional experiments are being conducted to test this interesting possibility.

The present studies reveal the recirculation of mature T cells back to the avian thymus. Beginning \( \sim 8 \) d after transplantation of the chick thymus, roughly the time at which chick T cells appeared in the quail spleen, T cells of chick origin could be identified in the thymus of the quail embryo. These immigrant T cells were confined to the medullary compartment of the host thymus. The migration of rare peripheral T cells to the thymus medulla has also been observed recently in mice (25). While elucidation of the physiological significance of this thymus re-homing pathway will require further experimentation, these observations clearly indicate that direct intrathymic maturation is not the only source of mature T cells in the thymic medulla.

During normal chick development, a population of lymphoid cells with an unusual phenotype, cytoplasmic CD3+surface CD3-TCR-CD4-CD8- (26), appears in the peripheral lymphoid tissues on E8. This lymphocyte subpopulation increases numerically as a function of embryonic age. It is therefore particularly noteworthy that no CD3+ cells were observed in the peripheral lymphoid tissues of the thymus chick-quail chimeras before the peripheral seeding of TCR-1+ cells. This finding supports the conclusion that this subpopulation of cytoplasmic CD3+ cells represents a thymus-independent lineage of cells.

**Summary**

Chick-quail chimeras were used to study precursor/progeny relationships of hemopoietic stem cells (SC) that enter the embryonic thymus in waves to give rise sequentially to the TCR-1+, TCR-2+, and TCR-3+ lineages of T cells. The first wave of SC and their progeny were examined by grafting thymus from 9-d chick embryos (E9) into E3 quails. mAbs specific for chick T cell antigens were used to trace the development of T cells in the recipients. All three lineages of TCR-bearing cells were generated from the first wave of SC. The cortico-medullary transit time was several day shorter for the TCR-1 subpopulation than for the TCR-2 subpopulation, and the peripheral seeding of TCR-2 cells also occurred later in development. The duration of thymocyte production from the first wave of SC that entered the thymus was \( \sim 3 \) wk, during which gradual cortical to medullary replacement by second wave SC
FIGURE 5. Continued on facing page.
FIGURE 5. Immunoperoxidase staining of chick thymus transplant at donor age of E27. Primary mAb: (A) CT3; (B) TCR1; (C) TCR2. Methyl Green counterstain (original magnification, x400).

FIGURE 6. Frequency of chick CT3+ cells in quail recipient spleen, bursa, and intestine. The frequency of total cells in each tissue was determined by nuclear counts (methyl Green counterstain).

FIGURE 7. Comparison of the ontogeny of TCR-1 (A) and TCR-2 cells (B) in the normal chick spleen (open symbols) and in the spleen of a chick-quail chimera (closed symbols). The embryonic age indicated for the chimera is that of the chick thymus implant. Cells identified by immunoperoxidase staining and expressed as a percentage of total cells identified by the methyl Green nuclear counterstain.
FIGURE 8. Continued on facing page.
progeny occurred. When the latter was examined, after transplantation of E7 quail thymus into E3 chick embryos, a sequential generation pattern for the TCR-1 and TCR-2 cell progeny was not evident. Finally, recirculation of T cells to the thymus medulla was defined in this avian model.

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