IDENTIFICATION OF A T3/T CELL RECEPTOR COMPLEX IN CHICKENS

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Closely associated with the antigen receptor molecule on the surface of human T cells is the T3 complex composed of two glycoproteins with M_r of 25,000 (γ chain) and 20,000 (δ chain), and a nonglycosylated protein of M_r 20,000 (ϵ chain) (1, 2). Murine mAb have been made that recognize the human T3 molecule. Physical association of the T3 molecule with the T cell receptor (TCR) has been suggested by the ability of anti-T3 antibodies to coprecipitate α and β chains of the TCR (3, 4). mAb directed against either T3 or the TCR induce the same T cell activation signal (5, 6).

Because of its importance in human T cell function, we anticipated that the T3/TCR complex would prove to be phylogenetically conserved. Recently (7–9), a murine T3 candidate has been identified by its coprecipitation with TCR α and β chains. Here we report evidence indicating the existence of a functional T3/TCR complex in birds.

Materials and Methods

Lymphocyte Isolation and Immunofluorescence. Outbred white leghorn chickens, eggs, and young Indiana giant quails were obtained from local sources. Isolation of mononuclear cells from blood, thymus, bursa, spleen, and bone marrow, and immunofluorescent staining were as described (10, 11). The frequency of stained cells was analyzed by fluorescence microscopy or automated flow cytometry. Capping of lymphocyte surface molecules after immunofluorescent staining was evaluated after incubation of stained cells for 30 min at 37°C.

Preparation of mAb CT-3. BALB/c mice were immunized by subcutaneous and footpad injections with chicken thymocytes and Ig⁻ blood mononuclear cells, and their lymph node cells were fused with P3-X63Ag8.653 myeloma cells (11). One resultant hybridoma produced an IgG1ₖ antibody, CT-3, showing immunofluorescence reactivity with a subpopulation of blood lymphocytes. The hybridoma was subcloned, grown in ascites, and the CT-3 antibody was purified on a protein A–Sepharose 4 B column. For mitogenicity studies, the purified CT-3 antibody was conjugated to Sepharose 4 B beads, and the exact amount of CT-3 bound to the beads calculated.

Immunoprecipitation and Gel Electrophoresis. Blood mononuclear cells were surface-labeled with Na¹²⁵I using the lactoperoxidase method, and lysed in either 1% NP-40 or 1% digitonin plus 0.2% NP-40 in 0.05 Tris, pH 7.5, containing protease inhibitors, at 4°C for 30 min (9, 10). Immunoisolation was performed in a microtiter plate by a solid-
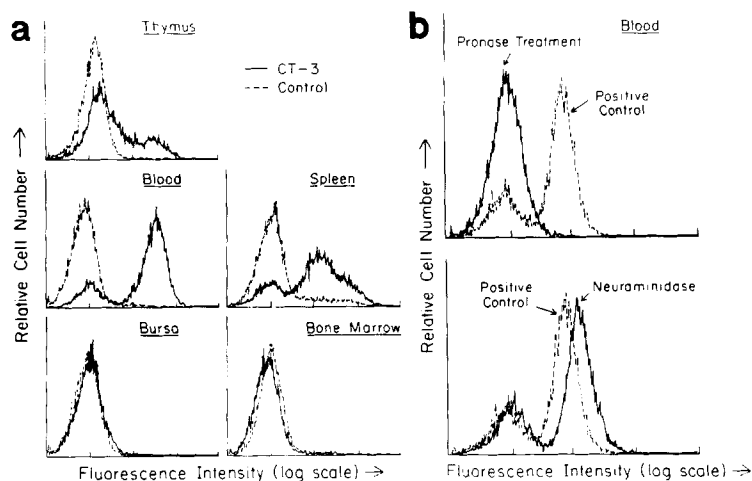
FIGURE 1. *a*. Immunofluorescence analysis of cellular reactivity with the CT-3 mAb. The results are presented as fluorescence histograms with the relative fluorescence intensity on a logarithmic scale. Solid lines indicate the staining intensity of the cell-bound CT-3. Dashed lines indicate staining of cells with a control IgG1 mAb. *b*. Immunofluorescence analysis of the effect of enzyme treatment on CT-3 expression on blood mononuclear cells. Dashed lines indicate immunofluorescence intensity of cell-bound CT-3 before enzyme treatment, and solid lines indicate the staining intensity after pronase or neuraminidase treatment. The effect of trypsin treatment (not shown) was identical to that of pronase.

phase method (11). CT-3-reactive molecules were dissociated from the plate by incubation with 2% SDS with or without 2-ME (reducing or nonreducing conditions) at 37°C for 1 h, and examined by SDS-PAGE in the Laemmli system.

Enzymatic Treatment. Blood mononuclear cells were treated wtih 1 mg/ml trypsin, pronase, or neuraminidase at 37°C for 30 min as described (11). For endoglycosidase-F treatment, the ^{125}I-immunocomplex was dissociated from microtiter wells by incubation with 100 mM Tris-HCl, pH 6.8, containing 1% SDS and 10 mM EDTA at 80°C for 20 min. CT-3-reactive molecules were then digested with endoglycosidase-F at 37°C for 4 h (9).

[^{3}H]Thymidine Incorporation. Blood mononuclear cells were incubated with the stimulating agent in RPMI-1640 culture medium containing 5% FCS in flat-bottomed microtiter plates at 37°C in a humdified atmosphere containing 5% CO_2. After 3 d, the cells were pulsed with 1 μCi [^{3}H]thymidine for 18 h, and the samples were harvested using a semiautomatic cell harvester. [^{3}H]Thymidine incorporation was measured with a scintillation counter.

Results

Tissue Distribution of Lymphocytes Reactive with the CT-3 Antibody. Indirect immunofluorescence analysis revealed that CT-3 antibody reacted with ~30% of thymocytes, 70% of blood mononuclear cells, and 60% of splenocytes, but <1% of cells in the bursa and bone marrow (Fig. 1*a*). When blood mononuclear cells were surface stained either with CT-3, the M-4 anti-μ mAb (10), or CT-3 plus M-4, 70, 23, and 93%, respectively, of the cells stained. This suggested that CT-3$^+$ cells and IgM$^+$ cells belong to separate lymphocyte populations, a conclusion that was confirmed when cells were examined by two-color immunofluorescence for reactivity with CT-3 or M-4. CT-3 was also found to react with a T cell line (MDCC-Cu15/40), but not with the B cell lines (BK25, 249L$_4$, 243L$_1$,
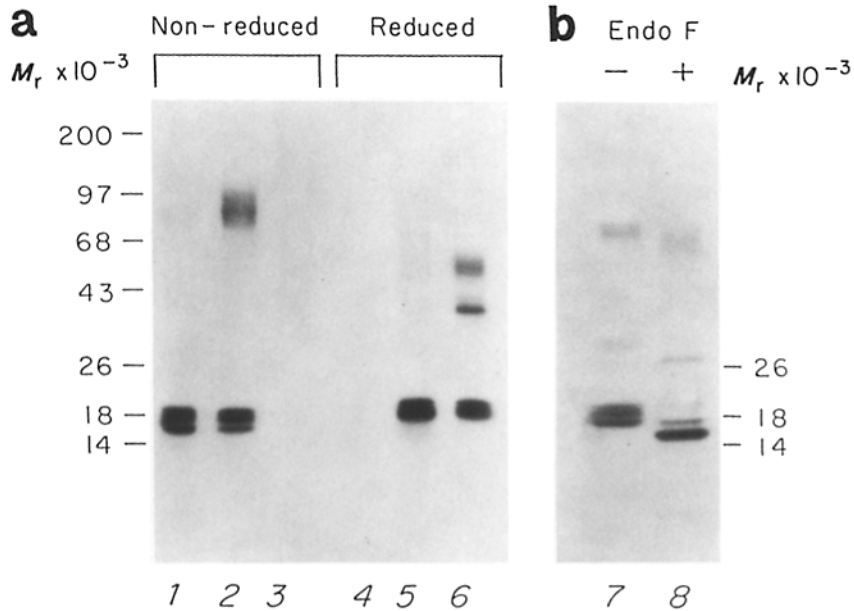
FIGURE 2.   a. CT-3-reactive surface proteins analyzed by SDS-PAGE. ^{125}I–surface-labeled blood mononuclear cells were solubilized with NP-40 (lanes 1, 4, 5) or digitonin (lanes 2, 3, 6). Immunoprecipitates were prepared with CT-3 (lanes 1, 2, 5, 6) or a control mAb (lanes 3, 4) and analyzed on a 5–20% gel under nonreducing (lanes 1–3) or reducing (lanes 4–6) conditions. b. Endoglycosidase-F digestion of CT-3-reactive proteins. ^{125}I-labeled immunoprecipitates prepared with CT-3 were digested with endoglycosidase-F (lane 8). Undigested CT-3 immunoprecipitates were run in parallel (lane 7).

293B$_9$) or a myeloid cell line (BM2) tested. CT-3 antigens were capped after crosslinkage by the antibody, suggesting that CT-3 reacts with a surface molecule on chicken T cells that has transmembrane connection to intracellular contractile elements. Quail lymphocytes, on the other hand, were not reactive with CT-3.

Biochemical Characterization of CT-3-reactive Molecules.   Treatment of chicken T cells with trypsin or pronase abolished CT-3 immunofluorescence reactivity, while neuraminidase treatment increased the immunofluorescence intensity slightly (Fig. 1b). SDS-PAGE analysis of the CT-3-reactive molecules isolated from NP-40-solubilized T cells revealed three bands of M_r 20,000, 19,000, and 17,000 under nonreducing conditions (Fig. 2a, lane 1). Under reducing conditions, the apparent sizes were not altered significantly (lane 5). Removal of N-linked sugars from the isolated CT-3-reactive molecules by endoglycosidase-F treatment yielded two bands of M_r 17,000 and 16,000 (Fig. 2b, and lane 8). These results suggest that the M_r 20,000 and 19,000 proteins were deglycosylated and migrated as a single band of 16,000, while the M_r 17,000 protein was unaffected. Identical results were obtained when the CT-3-reactive molecules on thymocytes and the Cu15/40 T-cell line were examined.

When ^{125}I-labeled avian T cells were solubilized with digitonin, CT-3 coprecipitated a polypeptide of M_r 92,000 under nonreducing conditions (Fig. 2a, lane 2), and two polypeptides of M_r 49,000 and 37,000 under reducing conditions (Fig. 2a, lane 6).

TABLE I

CT-3 Antibody Induces DNA Synthesis

Stimulus of blood mononu-clear cells	[³H]Thymidine incorporation (cpm) at stimulus concentrations (μg/ml) of:					
	0	0.1	0.5	1.0	2.5	10
CT-3 (soluble antibody)	322*	3,193	4,152	5,981	6,104	ND
CT-3-Sepharose beads	322	6,197	21,645	38,854	18,422	ND
Control Sepharose beads	322	771	801	546	927	ND
Con A	322	ND	ND	ND	ND	34,405

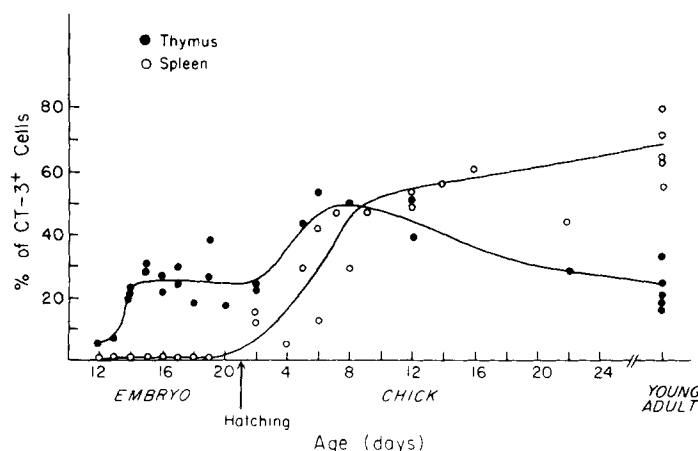* Numbers represent [³H]thymidine incorporation by 10⁶ cells from triplicate cultures.

FIGURE 3. Ontogeny of CT-3⁺ thymocytes (●) and splenic cells (○) detected by cell surface immunofluorescence. Cell pools derived from at least one dozen embryos were used for each time point during the embryonic period. Cells from one chicken were used for each point after hatching.

Mitogenicity of CT-3 Antibody. Mitogenic activity of the CT-3 antibody on blood mononuclear cells was examined by [³H]thymidine uptake. CT-3 conjugated to Sepharose 4 B beads induced a vigorous proliferative response similar to that induced by Con A, while soluble CT-3 appeared to have a marginal mitogenic effect on T cells (Table I). An unrelated IgG1 mAb conjugated to the beads had no demonstrable effect.

Ontogeny of CT-3 Expression. The expression of CT-3-reactive molecules on thymocytes and splenocytes was examined by immunofluorescence microscopy from embryonic day 12 onward. The CT-3 antigen was present on ~5% of thymus cells from 12-d chick embryos. The frequency of CT-3⁺ thymocytes increased to 25% on day 14, and remained relatively constant thereafter until hatching on day 21. After hatching, the frequency of CT-3⁺ thymocytes increased to 50% over the first week of life, then decreased to 20–30% throughout young adulthood (Fig. 3). Splenic CT-3⁺ lymphocytes were occasionally observed in embryonic life, but their frequency remained at <1% until hatching. The frequency of CT3⁺ splenocytes progressively increased thereafter to reach 65% in young adults.

Discussion

These studies provide the first evidence for existence of a T3/TCR complex in a nonmammalian vertebrate. The avian T3 molecules consist of a complex of three polypeptides of M_r 20,000, 19,000, and 17,000, two of which are N-glycosylated. Thus the chicken T3 complex appears remarkably similar to the T3 molecular complex in humans, which consists of two N-glycosylated polypeptides and a nonglycosylated polypeptide (1, 2). The human T3 ϵ polypeptide is resistant to cell surface labeling with ^{125}I, but it can be radioiodinated via a hydrophobic labeling agent, and can be biosynthetically labeled (1). In contrast, the candidate T3 molecules in mouse consist of four or more polypeptides, all of which can be radioiodinated by the lactoperoxidase method (7–9) as is the case for the chicken T3 polypeptides. Further studies are needed to identify homologies of the chains among the different representative species.

The human T cell receptor was originally recognized with anticlonotypic antibodies (6, 12). Its α (M_r 44,000) and β (M_r 37,000) chains could also be identified as minor components in anti-T3 immunoprecipitates isolated from iodinated cell lysates (3, 4). Using an improved extraction procedure (9), CT-3 coprecipitated an polypeptide of M_r 92,000 from T cell lysates, which could be reduced to two polypeptides of M_r 49,000 and 37,000. These results suggest the existence of a chicken TCR homolog containing a disulfide-linked heterodimer of α and β chains associated with T3 molecules on the T cell surface.

The T cell mitogenic effect of the CT-3 antibody on Sepharose beads provides further evidence that the antibody identifies the avian counterpart of the mammalian T3/TCR complex. The marginal effect of soluble CT-3 is probably due to low binding affinity of avian Fc receptors for mammalian Ig (13), as interactions of anti–human T3 antibodies with monocyte Fc receptors are important in their mitogenic effects on T cells.

Hemopoietic stem cells colonized the avian thymic rudiment in waves, the first of which is attracted to the epithelial thymus in 6.5-d-old chick embryos (14). Expression of cell surface antigens marking T-lineage differentiation begins 5–6 d later, 80% of embryonic thymocytes expressing the thymus specific antigen CT-1 by the 14th embryonic day (11). The CT-3 antibody was reactive with ~5% of thymocytes on the 12th day of egg incubation. By day 14, 25% of embryonic thymocytes expressed CT-3 molecules, and the M_r 92,000 heterodimer could be detected in the CT-3 immunoprecipitate (data not shown). The data suggest that a 5–6-d interval of intrathymic sojourn is required for hemopoietic stem cell progeny to reach the stage of T3/TCR expression, and another week of intrathymic residence before the CT-3$^+$ cells begin to migrate to the spleen in significant numbers.

The CT-3 antibody, which identifies the T3 complex on chicken and not quail T cells, should be an ideal probe for examining the role of the T3/TCR complex in the development and function of T cells.

Summary

A mouse mAb, CT-3, recognizes on chicken T cells a complex of three polypeptides, M_r 20,000, 19,000, and 17,000, two of which are N-glycosylated. The CT-3 antibody is mitogenic for chicken T cells, and it coprecipitates two

additional polypeptides of M_r 49,000 and 38,000 in lysates of T cell membranes. Ontogeny studies revealed that 5–6 d after thymic influx of hemopoietic stem cells, their thymocyte progeny begin to express the T3/TCR complex. After hatching 1 wk later, the CT-3$^+$ cells begin splenic migration in large numbers.

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