

A Monoclonal Antibody against Chicken Thrombocytes Reacts with the Cells of Thrombocyte Lineage

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ABSTRACT. A new mouse monoclonal antibody (mAb), HUKT was raised against chicken peripheral blood thrombocytes. The mAb HUKT appeared to detect a specific marker on the surface of chicken thrombocytes. Flow cytometry (FCM) analysis revealed that it did not react with cells from the normal thymus, bursa of Fabricius, six kinds of chicken cell lines, chicken erythrocytes or human platelets. In addition, HUKT⁺ cells in peripheral blood leukocytes (PBL) were CD45^{low}, Bu-1a⁻ and CD3⁻ cells. Immunoblotting analysis showed that the molecule recognized by HUKT is a monomer with an apparent molecular weight of 150 kDa under non-reducing and reducing conditions. Tissue distribution studies revealed that only cells of thrombocyte lineage in bone marrow and embryonic blood cells were stained by HUKT. The HUKT mAb presented here may be useful for both ontogenetic studies of thrombocyte lineage and immunological studies in the chicken.

KEY WORDS: chicken, mAb, ontogeny, thrombocyte.

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Chicken thrombocytes are nucleated blood leukocytes homologous in function to mammalian platelets. They represent the most numerous white blood cell types in chicken blood and perform a hemostatic role similar to that of platelets [5, 6]. Unlike mammalian platelets, they are nucleated cells, similar in specific gravity and size to small lymphocytes. They can be distinguished morphologically from monocytes and lymphocytes by electron microscopy and in fresh preparations by phase contrast microscopy, but may be mistaken for small lymphocytes using conventional microscopy. When chicken blood is centrifuged, the thrombocytes separate out with the leukocytes into the buffy coat. The actual number of thrombocytes appears to be about the same as that of total leukocytes. It remains very difficult to discriminate between lymphocytes and thrombocytes according to cell size and cellular construction using flow cytometry (FCM) analysis. In addition, chicken thrombocytes have not been studied in detail, because of the lack of thrombocyte-specific markers.

The origin of the mammalian platelet is the giant cell, the megakaryocyte of the lungs and bone marrow. The megakaryocyte is lacking in bone marrow of the chicken and the thrombocytes arise from antecedent which were mononucleated cells and have a blast stage like other cells [15]. In addition, Archer [2] stated that large cells in chicken bone marrow are not comparable to mammalian megakaryocytes, they appear to be multinucleated and could be the precursors of thrombocyte. However, these presumptions remain to be confirmed.

Monoclonal antibody (mAb) reactive with differentiation antigens is an excellent tool for the identification and characterization of various cells and cell lineage. However, in contrast to mammalian platelets, relatively few mAb specifically reactive with the thrombocyte lineage in chickens have been described. Some mAbs reactive with the thrombocytes are cross-reactive with other chicken cells [9, 20]. Only one mAb, 11C3, raised against chicken PBL is identified as a cell-specific marker, which is detected on the surface of chicken thrombocytes [13]. However, glycoprotein IIb-IIIa (GPIIb-IIIa) recognized by 11C3 mAb was expressed on chicken multilineage hematopoietic progenitor cells, e.g. myeloid, erythroid and T-cell progenitors [17].

In this paper, we describe a new mAb, HUKT, which has specificity restricted to cells of the thrombocytic lineage in chickens. The molecule recognized by HUKT is different from that recognized by 11C3. The HUKT mAb presented here may be useful for the studies of thrombocyte lineage.

MATERIALS AND METHODS

Animals: Congenic chickens (H-B15 White Leghorn) supplied by Dr. Vainio (Department of Medical Microbiology, Turku University, Finland) were used in this study. These chickens were bred in our animal facilities, and provided with feed and chlorinated water *ad libitum*. Eggs derived from H-B15 chickens were incubated at 38°C in a humidified incubator until the desired developmental age was reached. Female BALB/c mice were 8 weeks old, and were purchased from SLC (Hamamatsu, Japan).

Cell lines: The chicken cultured cells, lymphoid leukemia lymphoblastoid B cell line (1104B1) [10], macrophage cell line (HD11) [3], monocytic leukemia cell line (IN24) [12]

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and three Marek's disease lymphoblastoid T cell lines (MSB1 [1], JP2 [28] and RP1 [16]) were grown in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum in a 5% CO₂ incubator at 38.5°C.

Cell preparations: Blood was collected from the wing vein on 77 mM EDTA-PBS (pH 7.4). Thrombocyte-enriched PBL were prepared by the method previously described [11]. All surfaces coming in contact with the blood or cell suspension were siliconized except plastic instruments. Collected blood was mixed with 7.7 mM EDTA-PBS (pH 7.4) at a 1:1 ratio. The blood was then layered on a Ficoll-Paque density gradient (Pharmacia Biotech, U.S.A.) and centrifuged at $1,700 \times g$ for 25 min. The cells recovered from interface were washed three times with PBS and used as a thrombocyte-rich preparation. The cell suspension was composed of about 90% thrombocytes and very few numbers of lymphocytes [11]. For isolation of PBL, buffy coat cells were collected from whole blood by centrifugation at $700 \times g$ for 10 min. PBL were isolated by centrifugation of the buffy coat cells over a Ficoll-Paque density gradient (Pharmacia Biotech, U.S.A.) at $700 \times g$ for 10 min. The interface cells were collected in Hanks' balanced salt solution (HBSS) and washed three times in HBSS. Erythrocytes were obtained from whole blood by centrifugation at $700 \times g$ for 10 min and the buffy coat cells were removed before washing three times in HBSS. Bursa of Fabricius, spleen, and thymus cell suspensions were prepared by gentle homogenization of the organs in HBSS. Bone marrow cells were harvested by flushing tibias and femurs with HBSS and passing the cells through a 21-gauge needle. The organ cells were separated from dead cells and erythrocytes on a Ficoll-Paque density gradient. Cells were washed three times in HBSS. Cell viability, as determined by trypan blue exclusion, was greater than 90% for all cell preparations.

Antibodies: Monoclonal antibodies used in this study were HUKT, an IgG1 mAb described herein, and monoclonal anti-chicken CD3 and CD45 that were purchased from Southern Biotechnology Associates, Inc., (U.S.A.). Monoclonal antibody L22 (mouse IgG1) [26] against Bu-1a was a generous gift from Dr. Vainio (Turku University, Finland), and used as a chicken B cell marker.

Production of mAb HUKT: BALB/c mice were immunized with 1×10^8 chicken thrombocytes in 250 μ l of PBS. Four intraperitoneal injections were given at 3 weeks intervals. Three days after the last injection, the spleen cells of these mice were fused with SP2/0-Ag14 mouse myeloma cells as described [21]. Hybridomas were screened by immunofluorescence for their reactivity on thrombocytes, as well as bursal cells, splenocytes, thymic cell populations, and erythrocytes. The heavy chain isotype of the mAb was determined using the mouse monoclonal sub-isotyping kit (American Qualex, U.S.A.).

Purification and conjugation of monoclonal antibodies: The ascitic fluids obtained from mice inoculated with each hybridoma were precipitated with ammonium sulfate. Immunoglobulin fractions were separated using Sephacryl S-200 HiPrep (Amersham/Pharmacia, Japan), equilibrated

with 0.1 M NaH₂PO₄·12H₂O (pH 7.2), 0.2 M NaCl, and concentrated. Biotinylated antibodies were prepared according to standard methods [8]. Briefly, purified monoclonal antibodies (1 mg/ml) were dialyzed against 0.1 M sodium borate buffer pH 8.8, and reacted with 10 mg/ml biotin N-hydroxysuccinimide ester (Pierce, IL) in DMSO (250 μ g for 1 mg of antibody) for 4 hr. Reactions were stopped by the addition of 1 M NH₄Cl (20 μ l for 250 μ g of biotin ester) for 10 min. Conjugated antibodies were purified using a Sephacryl S-200 column.

Immunofluorescence staining: For indirect immunofluorescence staining, the cells were incubated with HUKT mAb for 30 min at 4°C. The cells were washed three times with HBSS supplemented with 1% bovine serum albumin (BSA) and 0.1% NaN₃ (washing buffer). The cells were then incubated with FITC-conjugated sheep anti-mouse Ig F(ab')₂ Fragment (Silenus, Australia) for 30 min. For the double staining of HUKT and anti-chicken CD3, anti-chicken CD45 or anti-Bu-1a, the HUKT-staining cells were blocked with normal mouse serum for 30 min at 4°C. After washing with washing buffer, the cells were incubated with biotin-labeled anti-CD3, anti-CD45 or anti-Bu-1a, respectively, for 30 min. After washing with washing buffer, the anti-CD3 reacted cells were incubated with streptavidin-conjugated phycoerythrin (PE) (Becton Dickinson, U.S.A.) for 30 min and then washed with washing buffer. After immunostaining, the dead cells were eliminated based on the propidium iodide incorporation.

Fluorescence staining was measured and positive cells enumerated by flow cytometry on a FACS Calibur (Becton Dickinson, U.S.A.). For each plot, analyses were made on 5,000 to 20,000 gated events, based on forward and side scatter parameters. The data was analyzed using CellQuestTM (FACS research software, Becton Dickinson, U.S.A.).

Western blotting analysis: Chicken thrombocyte-enriched PBL were disrupted in a lysis buffer [1% digitonin, 10 mM triethanolamine, 150 mM NaCl, 10 mM iodoacetamide, 1 mM EDTA, 10 μ g/ml aprotinin (pH 7.8)], and centrifuged at $100,000 \times g$. Their supernatants were mixed with a sample buffer with or without 2ME. Next, 10 μ l of samples were run in 5–20% polyacrylamide slab gel, and blotted to a Hybond-N (Amersham/Pharmacia, Japan) membrane. The membrane was blocked with alkaline phosphatase-free casein for 1 hr. The membrane was incubated with HUKT and then with alkaline phosphatase labeled goat anti-mouse IgG (Kirkegaard and Perry Lab., U.S.A.). CDP-Star chemiluminescent substrate (New England Bio Labs., U.S.A.) was used to provoke light emission.

Immunohistochemical staining: Smear preparations were made of bone marrow and blood that was collected with a glass capillary from the vitelline veins, the allantoic veins or heart of chick embryos from Day 3 of embryonic development (E3) until several weeks after hatching. The cell smears were fixed in acetone for 30 min at –20°C and immunostained as hereafter described.

For preparation of the sections from bone marrow, the

femur of chick embryos were collected from E9 until several weeks after hatching, embedded in OCT compound (Miles Inc., U.S.A.), frozen on dry ice, and stored at -80°C . Cryostat sections of $6\text{ }\mu\text{m}$ in thickness were air dried on slides treated with 0.0001% poly-L-lysine (Sigma, Japan) and fixed with cold-acetone for 10 min.

Air-dried smears and sections were rehydrated in PBS and incubated with 10% normal horse serum in PBS for 20 min to block the non-specific binding. Smears and sections were then incubated for 1 hr with HUKT diluted with 1% BSA. They were washed three times with PBS and incubated with biotinylated goat anti-mouse IgG (Vector Lab., U.S.A.), diluted with 1% BSA PBS for 30 min, and washed as before. Endogenous peroxidase was quenched with 0.3% H_2O_2 in methanol for 30 min, followed by incubation with ABC complex (Vector Lab., U.S.A.) for 30 min. Primary antibody-binding sites were finally immersed in freshly prepared substrate (0.05% 3'3'-diaminobenzidine tetrahydrochloride, 0.01% H_2O_2 , in 0.1 M Tris-HCl, pH 7.2). The smear and section were counterstained in hematoxylin. All incubations were performed at room temperature in a moist chamber. Control staining was carried out simultaneously, involving the first antibody being replaced with normal mouse IgG. No specific staining was found in the control slides.

RESULTS

Specificity of HUKT against various chicken cells: May-Gruenwald-Giemsa staining of chicken PBL showed that thrombocytes varied considerably in size, and that their shape varied from oval to round (Fig. 1A). The typical thrombocyte is oval, with a slightly oval nucleus in the center of a clear cytoplasm [4] and, as such, may be mistaken for small lymphocytes. HUKT mAb stained the surface of cells which were oval and contained an oval nucleus (Fig. 1B). The morphological criteria of HUKT-positive cells (HUKT⁺ cells) were identical to those of typical thrombocytes. We performed the same analyses on granulocytes and monocytes, and the cells were not positive (data not shown).

Single cell suspensions from different origins were stained with the HUKT mAb. The results were expressed as percentages of positive cells determined by indirect immunofluorescence, measured by flow cytometry (Table 1). The percentage of HUKT⁺ cells in thrombocyte-enriched PBL was $86 \pm 7.2\%$ [mean \pm standard deviation (SD), $n=5$]. Among the different organs tested, the percentage of HUKT⁺ cells detected in the spleen was low. No staining was apparent in the bursa of Fabricius, thymus, erythrocytes, and human platelet or in six kinds of chicken cell lines. Thus, the HUKT mAb appears to specifically recognize thrombocytes in chicken blood.

Chicken PBL analysis using HUKT mAb: PBL from five 3-week-old HB-15 chickens were analyzed according to flow cytometric light scatter. Profiles of bidirectional control maps from these samples resembled each other, and a

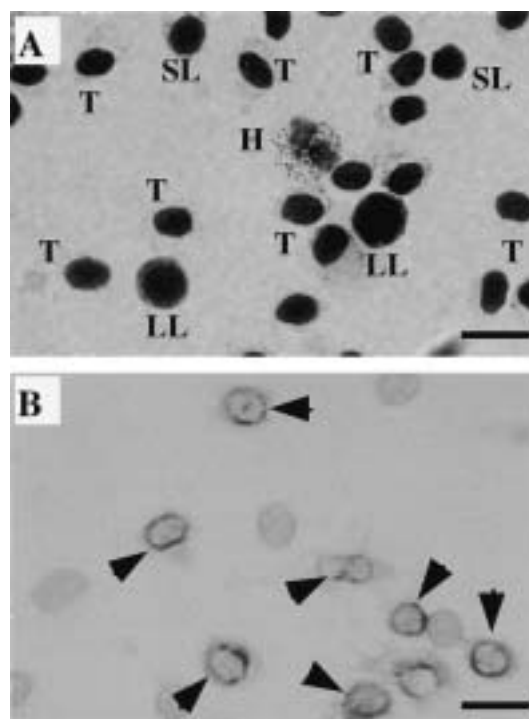


Fig. 1. May-Gruenwald-Giemsa staining (A) and HUKT immunostaining (B) of adult chicken PBL. (A) H, heterophil; LL, large lymphocyte; SL, small lymphocyte; T, thrombocyte (B) Arrowheads, HUKT⁺ cells (bar; $10\text{ }\mu\text{m}$).

Table 1. Reactions of HUKT mAb with live cells as determined by flow cytometry

Cell origin or cell type	HUKT-Positive cells (% \pm SD) ^{a)}
Thrombocyte enriched PBL (P21) ^{b)}	86 ± 7.2
Erythrocyte (P21)	0
Splenocyte (P21)	2 ± 1.1
Bursal cell (P21)	0
Thymic cell (P21)	0
Human platelet	0
Chicken cell lines	
MSB1 ^{c)}	0
JP2 ^{c)}	0
RP1 ^{c)}	0
1104B ^{d)}	0
HD11 ^{e)}	0
IN24 ^{f)}	0

a) Mean \pm standard deviation (SD), $n=5$.

b) Pn, number of days posthatching.

c) Marek's disease lymphoblastoid T cell line.

d) Lymphoid leukemia lymphoblastoid B cell line.

e) Macrophage cell line.

f) Monocytic leukemia cell line.

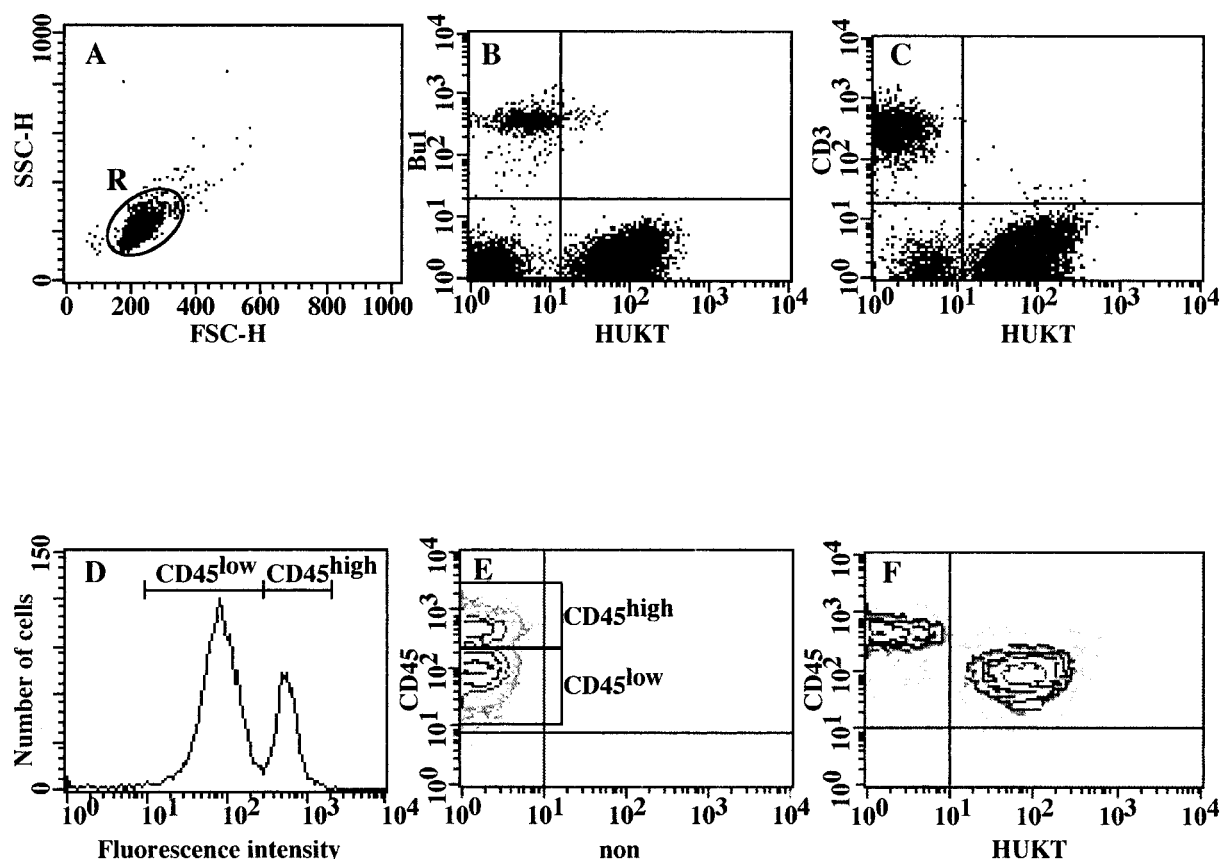


Fig. 2. Flow cytometric analysis of HUKT⁺ cells in chicken PBL. A: A bidirectional control plot of chicken PBL based on light-scatter characteristics of HUKT⁺, Bu-1a⁺ and CD3⁺ cells. B and C: Two-color staining with HUKT, and anti-Bu-1a (B) or anti-CD3 (C) in region 'R' shown in A. D and E: The fluorescence profiles of anti-CD45 mAb in region 'R' shown in (A). F: Two-color staining with HUKT and anti-CD45 mAb.

representative profile is shown in Fig. 2A. Based upon the FCM analysis of chicken PBL by Eerola *et al.* [7], we consider that the cell types encircled (R region) on the fluorescence profile in Fig. 2A are lymphocytes and thrombocytes. Subsequently, reactivities of HUKT, anti-Bu-1a and anti-CD3 mAb with these 'R region' cell types were examined. The fluorescence profiles of anti-Bu-1a and HUKT, or anti-CD3 and HUKT mAb reactions are illustrated in Fig. 2B and C. The percentage of Bu-1a⁺HUKT⁻ and CD3⁺HUKT⁻ cells was 5.0 ± 2 and $26.6 \pm 3\%$ ($n=5$), respectively (Fig. 2B and C). The percentage of Bu-1a⁺CD3⁻HUKT⁺ cells was $68.2 \pm 3.8\%$ ($n=10$, Fig. 2B and C). The percentage of Bu-1a⁺HUKT⁺ and CD3⁺HUKT⁺ cells was $<1\%$ (Fig. 2B and C). These results indicate that Bu-1a⁺CD3⁻HUKT⁺ population in chicken PBL was thrombocytes.

CD45 is the major membrane-associated PTPase⁵ on mammalian leukocytes and the common leukocyte antigen [24]. The mAb against chicken CD45 was developed by Paramithiotis *et al.* [18]. The staining of chicken PBL with anti-CD45 was biphasic with the vast majority of the cells falling into either a CD45^{high} or CD45^{low} population [18]. Paramithiotis *et al.* defined that CD45^{high} population was B-

and T-lymphocytes, and CD45^{low} population was non-lymphoid leukocytes. As shown in Fig. 2D and E, the fluorescence profiles of anti-CD45 mAb was biphasic as same as the result by Paramithiotis *et al.* [18]. The percentage of CD45^{high} and CD45^{low} cells was 73.1 ± 8.2 and $26.9 \pm 8.2\%$, respectively (Fig. 2D). Two-color staining analysis demonstrated that all peripheral CD45^{low} cells were HUKT⁺ cells (thrombocytes) (Fig. 2F). The CD45^{low}HUKT⁺ population did not react with anti-CD2, anti-CD4 and anti-CD8 mAb (data not shown).

Biochemical Characterization of the HUKT Antigen: To identify the thrombocyte membrane component(s) being recognized by the HUKT mAb, we performed western blotting analysis using detergent lysates from thrombocyte-enriched blood cells. The cells were lysed in the presence of digitonin. The results are presented in Fig. 3. A non-specific band of 60 kDa was observed at the reaction using normal mouse serum under non-reduced and reduced conditions (Fig. 3, lanes 1 and 2). HUKT mAb was immunostained as a single band with an apparent molecular weight of 150 kDa under non-reduced and reduced conditions (Fig. 3, lanes 3 and 4).

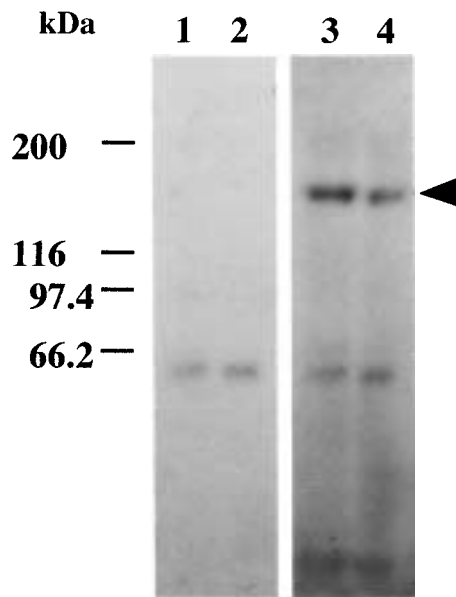


Fig. 3. Western blotting analysis of thrombocyte-enriched PBL lysates. Five μ l of lysates were subjected to electrophoresis on a 10% SDS-polyacrylamide gel under non-reducing (lanes 1 and 3) and reducing (lanes 2 and 4) conditions. Lanes 1 and 2 were stained with normal mouse antibody (IgG₁) as a control. Lanes 3 and 4 were stained with HUKT mAb. Arrow indicates the position of the HUKT-recognized molecule. The protein molecular weight markers are indicated on the left.

Reactivity of HUKT mAb with embryonic blood and bone marrow: The reactivity of HUKT mAb with embryonic blood by the immunohistochemical staining method is shown in Fig. 4. HUKT⁺ cells from embryonic blood began to be observed on Day 3 in embryonic (E3) blood. Large and round (8–11 μ m) HUKT⁺ cells identical to embryo thromboblats and/or medium embryo thrombocytes as previously described [23] were observed until E11. From E12 to E19, large and round HUKT⁺ cells were on the decrease, the typical thrombocytes in adult chickens increased (Fig. 4).

The reactivity of HUKT mAb with embryonic bone marrow by the immunohistochemical staining method is shown in Fig. 5. HUKT⁺ cells in embryo bone marrow were first observed on Day 12 in embryonic (E12) bone marrow. In the femur section on E10, the formation of cartilage was observed, but bone marrow or HUKT⁺ cells (data not shown). In the femur section on E12, a number of cells were observed in the central large space (bone marrow) (Fig. 5A), and HUKT⁺ cells were widely dispersed in the bone marrow (Fig. 5B). In the femur section on E19, the bone marrow was observed in the cartilage, and HUKT⁺ cells were observed as an assembly of cells in the bone marrow (Fig. 5D). The morphological criteria of HUKT⁺ cells on E12 and E18 were detected by the immunostaining of bone marrow

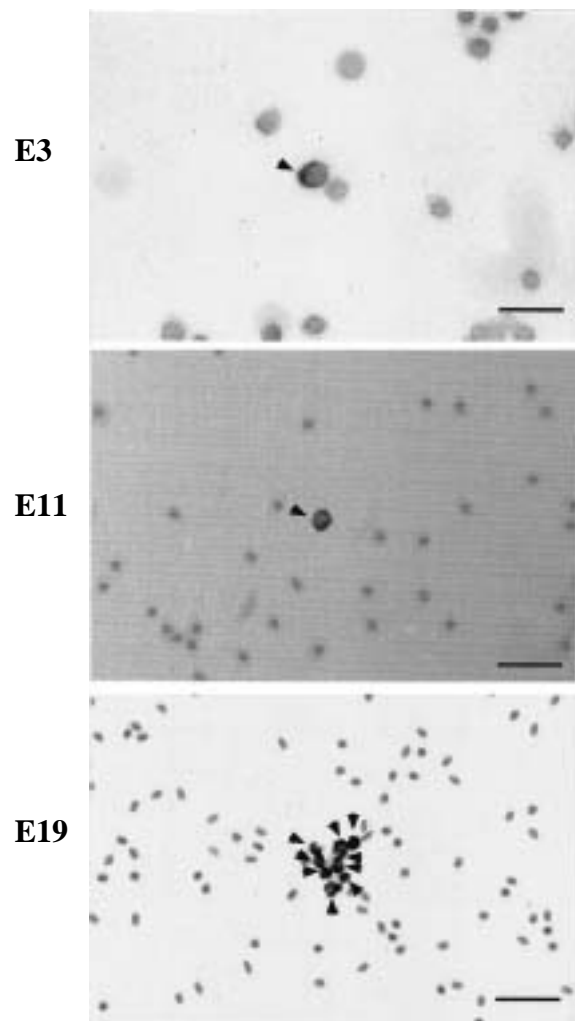


Fig. 4. HUKT immunostaining of embryonic blood smears on Day 3 of embryonic development (E3), E11 and E19. Arrows indicate HUKT⁺ cells. HUKT⁺ cells are almost erythrocyte or erythroblast (bar; 20 μ m).

smears. The HUKT⁺ cells were round and larger (7–11 μ m) than peripheral blood thrombocytes (6–9 μ m) (Fig. 5E, F). The HUKT⁺ cells that appeared in embryonic bone marrow were identical to thromboblats and/or early-immature thrombocytes as previously described [4].

DISCUSSION

This study describes development of a new mouse mAb, HUKT, raised against chicken thrombocyte-enriched PBL. HUKT mAb appears to be a specific marker detected on the surface of thrombocytes from the evidence provided by tissue distribution and FCM analysis. It did not cross-react with other chicken cell lines examined in this study.

Mammalian CD45 is a cell surface glycoprotein with a cytoplasmic tyrosine phosphatase domain that is believed to

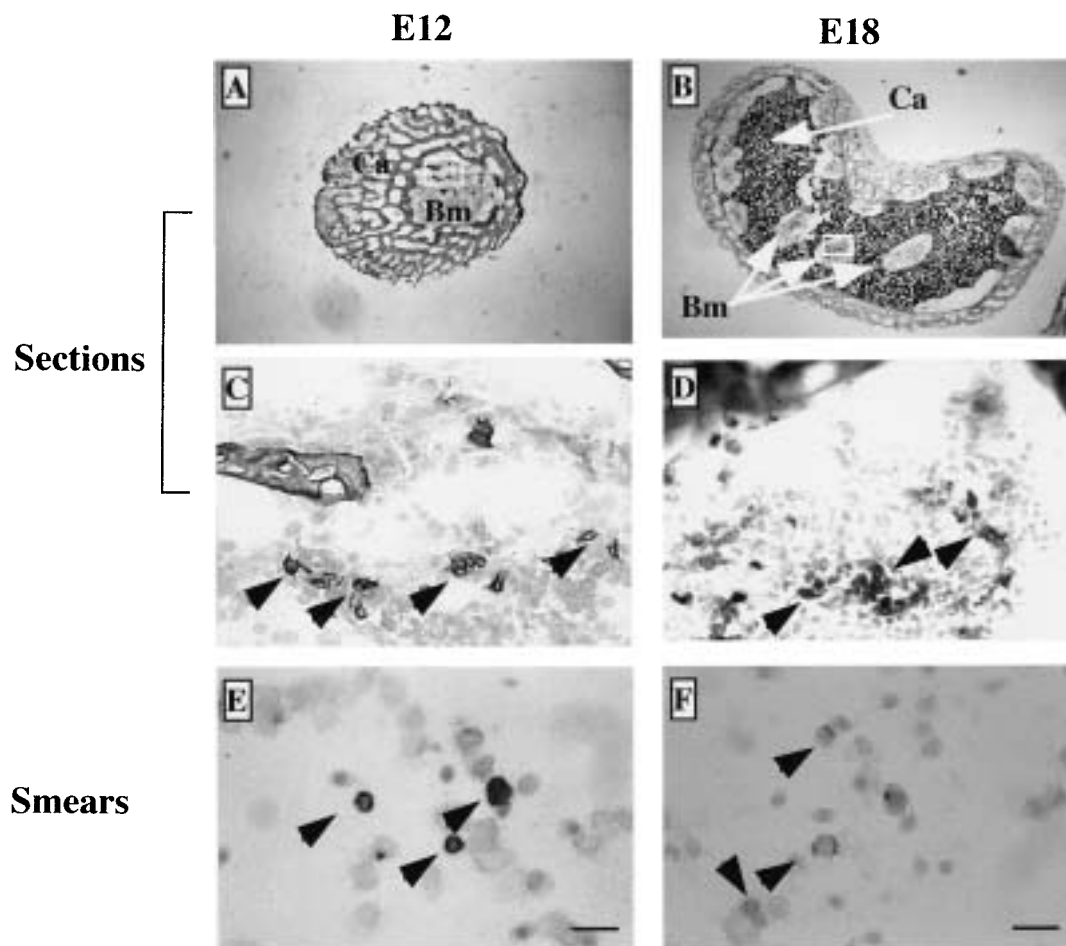


Fig. 5. HUKT immunostaining of embryonic femur section (A, B, C and D) and embryonic bone marrow cell smears (E and F). A and C, or B and D are the same section on E12 or E18, respectively (Magnification: A and B, $\times 29$; C, $\times 319$ and D, $\times 400$). Higher magnification from the field boxed in A and B was indicated C and D, respectively. Ca, cartilage; Bm, bone marrow. Arrows indicate HUKT⁺ cells (bar; 20 μ m).

play a role in T cell activation [25]. Various forms of CD45 are expressed on immature and mature leukocytes, including T and B-lymphocytes, thymocytes, mononuclear phagocytes, and polymorphonuclear leukocytes, except for mature erythrocytes and platelets [24]. On the other hand, chicken CD45 was expressed on all leukocytes but not on erythroid cells or their immediate precursors [24], and was used as leukocyte common marker in avian immunological studies. However, chicken CD45 has not been characterized so well as mammalian CD45. For example, immunostaining of chicken PBL with anti-CD45 mAb is observed biphasic peak (CD45^{high} and CD45^{low}) in FCM analysis (Fig. 2D and [18]). CD45^{high} population in chicken PBL was B- and T-lymphocytes, but the cell type of CD45^{low} population has been unknown. In this study, we defined that CD45^{low} population in chicken PBL was thrombocytes by double-immunostaining with anti-CD45 and HUKT mAb (Fig. 2F). Interestingly, CD45 is expressed on chicken thrombocytes

and mammalian megakaryocytes [19] but not on mammalian mature platelets. It is conceivable that CD45 expressed on peripheral blood nuclear cells in vertebrates and the developmental pathway of hematopoietic cells are different between chicken thrombocytes and mammalian platelets.

11C3 mAb [13] is the only previously known specific marker of thrombocytes. 11C3 mAb recognized the avian homolog of the mammalian platelet integrin and fibrinogen receptor GPIIb-IIIa. 11C3 mAb immunoprecipitates a heterodimeric molecule composed of two bands with an apparent molecular weight of 112 and 90 kDa under non-reducing conditions and of 112 and 26 kDa following reduction. On the other hand, the molecule recognized by HUKT was a monomer with an apparent molecular weight of 150 kDa under non-reducing and reducing conditions (Fig. 4). This evidence confirms that the molecule recognized by HUKT mAb differs from the one recognized by 11C3 and is, thus, another thrombocyte-specific molecule. When compared

with the membrane molecules of mammalian platelets, the HUKT mAb-recognized molecule might be analogous to platelet membrane protein, CD42b (GPIb beta), for the following reasons: the molecular weight (148 kDa) of CD42b [14] is similar to that of the HUKT mAb-recognized molecule (150 kDa) or because CD42b is one of platelet lineage-specific markers [27]. We attempted to determine the N-terminal amino acid sequence of the HUKT mAb-recognized molecule, but the sequence was undeterminable due to the blocked N-termini.

Specific mAbs against blood cells, acting as hematopoietic lineage markers, are a useful tool to study many developmental and functional aspects of blood cells in many animal species. However, chicken thrombocytes have not been studied in detail, partly because of the lack of lineage-specific markers. In our preliminary experiment, HUKT⁺ cells were observed in adult chicken bone marrow (data not shown). Therefore, we examined the reactivity of HUKT mAb with embryonic blood and bone marrow. HUKT⁺ cells began to appear on E3 in embryonic blood and on E12 in bone marrow (Figs. 2, 3). The HUKT⁺ cells in bone marrow and in embryonic blood from E3 to E12 revealed the typical shape and appearance of thromboblats and/or immature thrombocytes of various sizes [4, 23]. In addition, the HUKT⁺ cells appeared in the yolk sac on E2 (unpublished data). Tahara and Morinaka [22] detected the primary embryo thrombocytes in blood islands of chick blastoderms on E2. These results reveal the wide-ranging capability of HUKT mAb to detect cells of thrombocyte lineage. On the other hand, 11C3⁺ population in embryonic and adult bone marrow was myeloid, erythroid and T-cell progenitors [17]. The new mouse mAb, HUKT, and 11C3 mAb may be useful for the ontogenetic study on chicken hematopoietic cells including thrombocyte lineage.

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