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Expression of HIS50 Ag: a rat homologue of mouse heat-stable antigen and human CD24 on B lymphoid cells in the rat

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SUMMARY

Recently, a cDNA encoding a newly identified rat antigen (HIS50 Ag) that binds to monoclonal antibody (mAb) HIS50 was cloned and shown to be homologous to cDNA encoding murine heat-stable antigen (HSA) and human CD24. Here we show that, like CD24 and HSA, at least part of HIS50 Ag is inserted into the plasma membrane by a glycosylphosphatidylinositol (GPI)–lipid linkage and we describe its expression in rat haemolymphopoietic tissues. HIS50 Ag expression was almost exclusively confined to B lymphoid cells, the vast majority of T lymphoid cells, erythroid and myeloid cells were HIS50$^-$. Cell suspension analysis indicated that in bone marrow (BM) almost all Thy-1$^+$ cells, HIS24$^+$ cells [HIS24 recognizes the B-cell form of leucocyte common antigen (LCA)], terminal deoxynucleotidyl transferase-positive (TdT$^+$) cells and (c+s)κ^+ cells expressed HIS50 Ag, and all (c+s)μ^+ cells. A presumably early population of B lymphoid cells, expressing HIS24 Ag without HIS50 Ag, TdT or immunoglobulin (HIS24$^+$HIS50$^-$TdT$^-$ Ig$^-$), constituted 1·6% of BM nucleated cells. In blood, one-fifth of mononuclear cells were HIS50$^+$, and about 85% of these expressed μ and/or κ chains. In spleen, flow cytometry analysis and immunohistology demonstrated heterogeneous expression of HIS50 Ag: immunoglobulin M (IgM)^{bright} cells (as found largely in red pulp and marginal zone) were HIS50^{bright}, while IgM^{dull} cells expressed low or undetectable levels of HIS50 Ag. Germinal centre B cells expressed high levels of HIS50 Ag. Germinal centres of lymph nodes and tonsil of man also bound HIS50. We conclude that HIS50 Ag expression in the haemolymphopoietic system of rat is virtually restricted to the B lineage.

INTRODUCTION

Early development of B lymphocyte lineage cells from pluripotent stem cells to surface immunoglobulin (sIg) positive B cells is marked by successive rearrangement of the immunoglobulin gene loci, together with selective expression of surface located and intracellular markers (for review of B cell development in mouse see [1]).

In rat, pluripotent haemopoietic stem cells express high levels of the Thy-1-antigen.[2] This antigen is also expressed by virtually all B lineage cells in bone marrow (BM), *in vitro* granulocyte and macrophage colony-forming cells (GM-CFC), a few early erythroid precursors, early myeloid precursors and megakaryocytes.[2–6] Pluripotent haemopoietic stem cells do not express the high molecular form of leucocyte common antigen (LCA),[2] while this form of LCA detected by monoclonal antibody (mAb) HIS24 is expressed by the vast majority, if not all, B lineage cells.[3]

To further characterize early B lymphoid cell stages in rat on the basis of surface markers, we immunized mice with rat BM cells and produced monoclonal antibodies to B lineage cell surface antigens. The Ab HIS50 exhibited a binding pattern consistent with expression of the antigen (HIS50 Ag) early in the B lineage. We cloned a cDNA which, when expressed in COS cells encoded HIS50 epitopes.[7] The nucleotide sequence of this cDNA clone exhibited a high degree of homology with murine heat-stable antigen (HSA) and human CD24. The nucleotide sequence of the CD24 homologues predicts a glycosylphosphatidylinositol (GPI)–lipid linkage, a short mature peptide and extensive glycosylation, and these features have been confirmed at protein level for HSA and CD24.[8–9] Western blot data on HIS50 Ag also indicated a high degree of glycosylation, and many different glycoforms in organ-dependent patterns.[10] The exact function(s) of CD24/HSA are unknown at the moment; however, functions such as co-stimulation,[11–13] cell adhesion[14,15] and cell activation[16,17] have been implicated.

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Abbreviations: HSA, heat stable antigen; GPI, glycosylphosphatidylinositol; BM, bone marrow; LCA, leucocyte common antigen; TdT, terminal deoxynucleotidyl transferase; NCS, newborn calf serum; PLC, phospholipase C.

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In this paper we show that at least part of the HIS50-binding molecules are phosphatidylinositol (PI) linked and we describe the expression of HIS50 Ag within the B lymphoid lineage in BM and peripheral lymphoid organs.

MATERIALS AND METHODS

Animals, tissues and cell lines

Male PVG RT7^a rats were from stocks held at the department of Histology and Cell Biology, University of Groningen, male F344/Du rats were from stocks maintained at the University of Hong Kong; both were maintained under conventional conditions. Rats were killed at the age of 5–6 weeks or 3–4 months by an overdose of ether. Organs were dissected and processed as described below.

Rat cell line LAMA K-1 (early B lineage; 18) was from M. de Jonge and L. de Ley, Department of Clinical Immunology, University of Groningen, the Netherlands. LAMA cells were grown in RPMI-1640 (Sigma, St. Louis, MO) pH 7·4, supplemented with 20% fetal calf serum (FCS; Globe Pharm Ltd, Esher, Surrey, UK), with mouse or rat peritoneal cavity cells as feeders in the starting-up phase of the culture.

Human blood was drawn from healthy volunteers in the Department of Biochemistry, University of Hong Kong. Human tissues (frozen) were a kind gift of the Department of Pathology, University of Hong Kong (Head: Prof. F. Ho). Human cell lines Raji (pre-B lymphoma), BJAB (B lymphoma), Molt-4 (T leukemia) and HEL152 (embryonic lung fibroblast) were a kind gift from the Department of Microbiology, University of Hong Kong (Head: Professor M.H. Ng), and grown in RPMI-1640/15% FCS.

Antibodies

First stage antibodies. Polyclonal rabbit anti-terminal deoxynucleotidyl transferase (TdT) was from Supertechs Inc. (Bethesda, MD); mAb MRC OX19 (mouse immunoglobulin G1 (IgG1) anti-rat CD5[19,20]) was from the MRC Cellular Immunology Unit, Dunn School of Pathology; polyclonal rabbit anti-rat immunoglobulin was from Dakopatts (Glostrup, Denmark), mAb HIS8 (mouse IgG1 anti-rat κ chain[21]), HIS22 (mouse IgM anti-corona-type B lymphocytes of the rat[21]), HIS24 (mouse IgG2b anti-rat B220 (CD45R),[3,21] HIS40 (mouse IgG1 anti-rat μ chain[22]), HIS50 (mouse IgM anti-rat HIS50 Ag; clone 86.4.2D5; this paper) and HIS51 (mouse IgG2a anti-rat Thy-1[6]) were from the Department of Histology and Cell Biology, University of Groningen. mAb 331 (rat anti-mouse IgM[22]) conjugated to fluoroscein isothiocyanate (FITC) was a kind gift from Dr Aaron Kantor, Stanford University, CA.

Rabbit anti-TdT was diluted 1:10 in phosphate-buffered saline (PBS), rabbit anti-rat immunoglobulin was used diluted 1:1600 in PBS and all mAb were used as undiluted tissue culture supernatants.

Second stage antibodies. Goat anti-rabbit immunoglobulin(H+L)-conjugated to tetramethylrhodamine isothiocyanate (TRITC) or FITC, and goat anti-mouse immunoglobulin(H+L)–FITC were from Kirkegaard and Perry Laboratories (Gaithersburg, MD); goat anti-mouse Igγ1–TRITC or –FITC, goat anti-mouse Igμ–TRITC or –FITC, goat anti-mouse Igγ2a–TRITC and goat anti-mouse Igγ2b-TRITC or FITC were from Southern Biotechnology Associates (Birmingham, AL). Polyclonal peroxidase-conjugated rabbit anti-mouse Ig(H+L) Ab was from Dakopatts (Glostrup, Denmark). HIS40 conjugated to phycoerythrin (PE) was prepared by Dr Aaron Kantor, Stanford University, CA. Second stage antibodies were diluted to the appropriate working dilution with PBS containing 2–5% (v/v) normal rat serum. Just before use all antibodies were centrifuged at 15 000 ***g*** for 15 min.

Production of mAb HIS50

HIS50 was produced according to an immunization protocol described;[21] briefly, rat BM cells were used to immunize BALB/c mice, and SP2-0 myeloma cells served as fusion partner. Hybridoma culture supernatants were screened on BM cytocentrifuge preparations and femoral frozen sections using indirect immunofluorescent staining procedures.

Rat and human blood mononuclear cell samples

Blood drawn into heparin (50 U/ml) was diluted three-fold with RPMI-1640, layered onto Ficoll-paque (density 1·077 g/ml at 24°; Pharmacia, Uppsala, Sweden) (5 ml diluted blood per 2 ml Ficoll-paque) and centrifuged (100 ***g***, 25 min, room temperature). Cells were collected from the interface and washed twice with RPMI-1640 (400 ***g***, 10 min, room temperature (first wash), 200 ***g***, 5 min, 4° (second wash)).

Cells were resuspended in RPMI-1640/10% newborn calf serum (NCS; Globe Pharm Ltd, UK) to a concentration of 10^7/ml for immunostaining.

BM cell sampling

Both femoral shafts were flushed with 2 ml RPMI-1640 (Sigma, St Louis, MO), pH 7·4 supplemented with 10% NCS. After thorough suspension large particles were removed by 1 ***g*** sedimentation into 1 ml NCS (5 min, 4°). The upper layer was centrifuged through 1 ml NCS (10 min, 200 ***g***, 4°) and resuspended in RPMI/NCS to a concentration of 4×10^7 cells/ml.

Immunofluorescent staining procedures

BM and blood mononuclear cells and cell lines. All stainings were done sequentially with first stage Ab incubations for 1 hr, and second stage Ab incubations for 30 min. When cells were stained in suspension, they were kept throughout at 4°. In between incubation with each Ab and after the final Ab, cells were washed once over 2 ml RPMI/bovine serum albumin (BSA) (PBS containing 3% (w/v) BSA (Sigma)). Cytospin preparations and sections were stained and washed at room temperature. In between addition of each Ab the slides were washed three times for 10 seconds in PBS, after the last Ab, three times for 10 min. Immunofluorescent stained cytospins and sections were mounted in Citifluor (Citifluor, London, UK) to diminish fading of fluorescence. After sealing the coverslips with nail polish, the preparations were kept at 4° in the dark.

In all staining procedures appropriate positive and negative controls were included.

Double labelling for sHIS50 Ag versus (c+s) HIS50 Ag. BM and blood mononuclear cells (4×10^6 cells in 100 μl) or cells from the cell lines (1×10^6 cells in 100 μl) were incubated with HIS50, washed, resuspended in 100 μl RPMI/NCS, incubated with 100 μl goat anti-mouse Igμ–FITC and washed

again. The stained cells were cytocentrifuged and fixed in 5% (v/v) glacial acetic acid in absolute ethanol as described.[3] One hour after fixation the cells were incubated with 20 μl HIS50, followed by incubation with 20 μl goat anti-mouse Igμ–TRITC.

Double labelling for HIS50 Ag and HIS24 Ag or Thy-1. The labelling of BM cells for HIS50 Ag in suspension was followed by resuspension in 100 μl RPMI/NCS, incubation with 100 μl HIS24 or Thy-1 and, subsequently, goat anti-mouse Igγ2b–TRITC or goat anti-mouse Igγ2a–TRITC, respectively. The stained cells were cytocentrifuged and fixed as described.

Double labelling for HIS50 Ag and TdT, (c+s)μ or (c+s)κ. BM or blood mononuclear cells were cytocentrifuged and fixed as described above. One hour after fixation the cells were incubated with rabbit anti-TdT, HIS40 or HIS8, goat anti-rabbit immunoglobulin–FITC (TdT staining) or goat anti-mouse Igγ1–FITC (HIS40 and HIS8), HIS50, and finally goat anti-mouse Igμ–TRITC. Peripheral blood mononuclear cells from rat were stained with HIS50 plus goat anti-mouse Igμ–FITC in suspension, cytocentrifuged, fixed and stained with HIS8 or HIS40 plus goat anti-mouse Igγ1–TRITC.

Detection of $HIS24^+HIS50^-TdT^-Ig^-$ population. Basically, the procedure was as described above. HIS24 was labelled with FITC, HIS50, TdT and Ig with TRITC. BM cells were labelled with HIS24 and HIS50 while in suspension, and for TdT and immunoglobulin after cytocentrifugation and fixation.

Detection of HIS50 Ag in BM sections. Femurs were frozen and cross-sections of frozen femurs were stained as described.[6]

Immunoperoxidase staining of sections of frozen tissues

Tissues were snap frozen in liquid freon, 7 μm thick sections were cut in a cryostat (Harris or Bright), fixed in acetone (10 min), dried under a cool fan and stained using the immunoperoxidase technique.[21]

Microscopy and cell counts

Epifluorescence microscopes (Leitz, Wetzlar, and Zeiss, Standar 18, Germany) with appropriate filtersets for excitation and emission of FITC and TRITC, phase-contrast optics, and ×40 and ×100 oil immersion phase-contrast objectives were used for analysis. If not indicated differently, the number of cells analysed in each cytospin preparation was such that at least 100 cells positive for each marker were scored. Cell diameters were measured with an ocular micrometer, accurate to ±0·4 μm. Fluorescence photographs were made using a Leitz diaplan microscope equipped with a filter set appropriate for TRITC excitation and emission and a Leitz Orthomat E camera. Ektachrome 400 ASA film was exposed as 1600 ASA.

PI-specific phopholipase C treatment of cells

Rat BM cells were sampled as described above. Once resuspended in RPMI/NCS they were washed again through 2 ml PBS/BSA and resuspended in RPMI/BSA (RPMI-1640 supplemented with 0·2% (w/v) BSA pH 7·3) to a concentration of 4×10^7 cells/ml. *In vitro* cultured LAMA cells were harvested, washed twice in RPMI/BSA and resuspended in RPMI/BSA to a concentration of 2×10^7 cells per ml. PI-specific phospholipase C (PI–PLC) was from *Bacillus cereus* (Boehringer Mannheim Biochemica GmbH, West Germany). Cells were incubated (1 hr at 37° under 5% CO_2; after 30 min cells were resuspended by ticking the tube) in RPMI/NCS in a total volume of 200 μl per 1× (BM) or 0·5× (LAMA) 10^6 cells, containing 2·5 μl (0·125 U) PI–PLC in manufacturer's enzyme buffer (triethanolamine buffer, 50 mM; ethylene diamine tetraacetic acid (EDTA), 10 mM; sodium azide, 10 mM; pH 7·5) or 2·5 μl enzyme buffer (control). After incubation 2 ml of ice-cold RPMI/NCS was added, and cells were washed over PBS/BSA. After another wash the cells were resuspended in 100 μl RPMI/NCS and stained as described above with HIS50, OX1, OX7, HIS51, HIS24, HIS40 or medium (=control), followed by goat anti-mouse Igγ1–FITC, Igγ2a–FITC, Igμ–FITC or immunoglobulin(H+L)–FITC.

Flow cytometry analysis of cell suspensions

PI–PLC experiments. Stained enzyme and non-enzyme-treated cells were analysed in a Coulter Counter Profile II flow cytometer (Coulter Electronics Hong Kong Ltd, Hong Kong) by determining log-integrated green fluorescence of at least 5000 cells in each sample. Cells incubated with the second stage Ab only served as standard to adjust the photomultiplier tube-voltage so that less than 2% of the cells occurred within the 'positive' window. Then histograms of stained enzyme and non-enzyme-treated cells were recorded and compared by their mean fluorescence intensity in the 'positive' window.

BM, blood and spleen. Cell suspensions from BM, blood and spleen were prepared and resuspended at a concentration of 2×10^7 cells/ml in staining medium (Dulbecco's A+B/5%NCS/0·1% sodium azide). Half a million cells were incubated in round bottom microtitre plates with 25 μl HIS50 culture supernatants and mouse anti rat IgM (HIS40) conjugated to PE (30 min, at 4°). After washing with medium, cell suspensions were incubated with monoclonal anti mouse IgM (331) conjugated to FITC as second step reagent to detect HIS50 binding. Finally, cells were washed, resuspended in 200 μl staining medium and analysed with a dual laser Epics-Elite flowcytometer equipped with a gated amplifier (Coulter Electronics, Hialeah, FL) using Coulter software. Data were collected from at least 20 000 viable cells per sample. Dead cells and debris were excluded from the analysis using forward and sideward profiles.

RESULTS

mAb HIS50 was selected from screened hybridoma culture supernatants because it bound strongly to BM cells with lymphoid phase contrast morphology, while it did not detectably bind to BM cells of erythroid or myeloid morphology. The staining was very intense and discrete. In addition, immunofluorescent staining of cross-sections of frozen femur (Fig. 1) showed that HIS50 bound between one-third and one-half of the BM haemopoietic cells, while the remaining haemopoietic cells and the stroma appeared negative. Although HIS50-binding cells occurred all over the marrow, a high incidence of them was found near the endost, an area which is known to be rich in B precursor populations, such as TdT^+ cells and pre-B cells.[24] This suggested HIS50 Ag to be expressed early in the development of B lymphoid cells and therefore suitable to define early B lymphocyte precursor populations.

We investigated the mode of anchoring of HIS50-binding

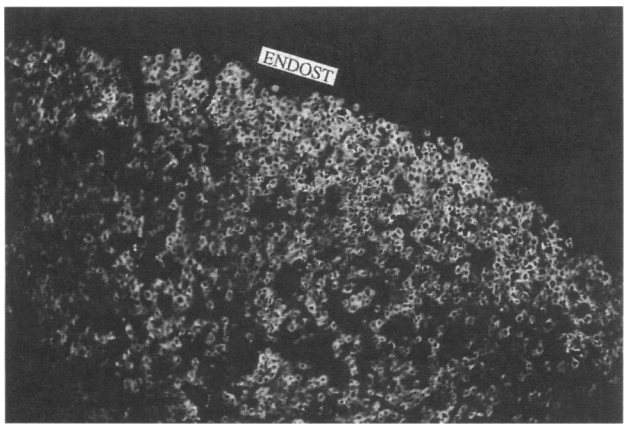

Figure 1. HIS50 expression in a cross-section of rat BM. HIS50$^+$ cells occur all over the marrow and at high density subendosteally.

molecules in the cell membrane. We characterized the HIS50-binding cells in BM and analysed HIS50-binding to other lymphoid and non-lymphoid organs in the rat. To assess evolutionary conservation of the HIS50 epitope we also analysed HIS50-binding to human tissues.

HIS50 Ag is anchored to the cell membrane through a GPI moiety

Both HSA and CD24 can be anchored to membrane lipids by covalent attachment to a GPI group.[8,9] Experimental evidence for this type of membrane anchorage is derived indirectly by the release of these molecules from the plasma membrane in the presence of PI-specific PI–PLC. We found that the density of HIS50 Ag on BM cells was reduced to half by incubation with PI–PLC (Fig. 2). A 10-fold increase in PI–PLC (1·25 U/10^6 cells) did not further reduce HIS50 Ag density (data not shown). The reduction profile showed no evidence of a PI–PLC resistant cell subset: all HIS50$^+$ cells had decreased

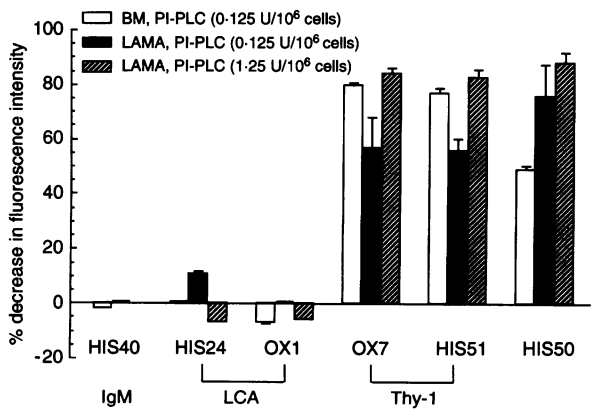

Figure 2. BM cells were incubated with or without PI–PLC and subsequently stained as described in Materials and Methods. (a) The decrease in fluorescence intensity was determined as 100% minus [(the mean fluorescence in the 'positive window' (see Materials and Methods) with PI–PLC treatment divided by the mean fluorescence in the 'positive window' without PI–PLC treatment) × 100%]. (b) For Thy-1 (OX7 and HIS51) the fluorescence intensity of BM cells actually decreased more than indicated due to a shift from the positive to the negative window of about one third of the cells.

fluorescence intensity. Under the same experimental conditions, Thy-1 (recognized by mAb OX7 and HIS51), a predominantly or exclusively GPI linked glycoprotein,[25] was also sensitive to PI–PLC. No change was observed in the surface abundance of the transmembrane protein LCA (recognized by mAb OX1 and HIS24) and of μ H chains (recognized by HIS40). Analysis of LAMA cells (an early B lineage tumour of the rat grown *in vitro*) gave similar results, PI–PLC treatment of these cells resulted in a 80–90% reduction of HIS50 Ag staining (Fig. 1). We conclude that, at least part of the HIS50-binding molecules is anchored to the cell membrane through a GPI–lipid linkage.

HIS50-binding B lymphoid cells in BM

The percentage of HIS50$^+$ cells in young rats was 39±2% of total nucleated BM cells (see Table 1). The intensity of HIS50 binding after fixation was much higher than that after binding to viable cells. This pattern of binding after fixation suggested the presence of a large intracellular pool of HIS50 Ag. Double-staining for s and (c+s) HIS50 Ag (see Materials and Methods) revealed that cells which expressed HIS50 Ag in the cytoplasm also expressed the Ag on the cell surface and that, if present, the frequency of cHIS50$^+$sHIS50$^-$ cells was below 0·1% (data not shown). To investigate which cells within the B lineage expressed the HIS50 Ag, BM and blood mononuclear cells were double-stained for HIS50 and a panel of B lineage-associated Ag (Table 1).

Thy-1

The vast majority of Thy-1$^+$ cells was HIS50$^+$ and vice versa. Of the Thy-1$^+$ cells that were HIS50$^-$ most were of lymphoid

Table 1. HIS50 Ag expression of BM and blood mononuclear cells in relation to other markers

Cell (sub) population	Percentage of all nucleated cells	Percentage HIS50$^+$	Percentage of HIS50$^+$ cells being
BM			
All nucleated	100*	39±2†	–
Thy-1$^+$	42±2	93±1	100±0
HIS24$^+$	38±3	95±1	98±1
TdT$^+$	2·4±0·1‡	95±1§	4·2±1·4
(c+s)μ^+	30±5	100±0	82±6
(c+s)κ^+	9·8±0·6	99±1	28±4
Blood mononuclear			
All nucleated	–	22±6	–
(c+s)μ^+	22±9	79±5	87±3
(c+s)κ^+	24±8	74±5	84±4

BM cells of 5–6-week-old male rats were double stained with HIS50 and one other Ab, and microscopically analysed for their HIS50-binding. The results are the mean of three animals.

*By definition.

†At least 500 cells of each subpopulation were analysed for HIS50-binding.

‡Due to the low incidence of TdT$^+$ cells only 25 TdT$^+$ cells were scored.

§Instead of 500, 200 TdT$^+$ cells were analysed for their HIS50-binding.

morphology, a few non-lymphoid Thy-1$^+$HIS50$^-$cells included megakaryocytes, macrophages (relatively large irregularly shaped cells with vesicles) and cells of erythroid morphology. The HIS50$^+$ cells which did not express Thy-1 bound HIS50 weakly. They were cells of myeloid morphology (doughnut-shaped nucleus and granules) and constituted less than 0·5% of BM nucleated cells.

HIS24

The vast majority of the HIS24$^+$ cells expressed HIS50 Ag, while virtually all of the HIS50$^+$ cells expressed HIS24. The HIS24$^+$50$^-$ population contained only cells of lymphoid morphology (see below). Among them were small cells mostly strongly expressing HIS24 Ag and larger cells that expressed HIS24 Ag in various intensities. The rare cells that were HIS24$^-$HIS50$^+$ were myeloid in phase contrast morphology.

TdT

Like cells bearing HIS24 Ag and Thy-1, the vast majority of the TdT$^+$ cells expressed HIS50 Ag. The cells which did not express HIS50 Ag expressed TdT weakly to strongly and varied in cell size from small to large.

μ^+ cells

All (c+s)μ^+ BM cells expressed HIS50 Ag; they constituted about 80% of the total HIS50$^+$ cells.

κ^+ cells

Virtually all (c+s)κ^+ cells showed HIS50-binding. A very small proportion ($\approx$0·1% of BM nucleated cells) expressed κ L chains, but no HIS50 Ag. Among these were plasma cells (abundant κ-containing cells with plasma cell morphology; total of 107 observed).

Thus, in summary, in BM HIS50 Ag is almost restricted to lymphoid cells and is expressed by HIS24,$^+$ Thy-1$^+$ and by most TdT$^+$ cells. These cells include pro-B and pre-B cells.

Characterization of HIS24$^+$HIS50$^-$TdT$^-$Ig$^-$cells

As mentioned above, a small percentage of BM cells was HIS24$^+$ but HIS50.$^-$ By analogy to mouse (B220$^+$HSA$^-$) this population may contain early stages of B lymphocytopoiesis. We determined its frequency and its cell size distribution. The HIS24$^+$HIS50$^-$TdT$^-$Ig$^-$ population comprised 1·6$\pm$0·0% of BM nucleated cells (mean$\pm$SD of three 6–5-week-old animals; for each rat at least 100 HIS24$^+$HIS50$^-$TdT$^-$Ig$^-$ cells were scored), all of lymphoid morphology. About 90% were weakly HIS24$^+$, the rest mostly moderately with a few strongly HIS24$^+$. The size distribution of this population is in Fig. 3. The HIS24$^+$HIS50$^-$TdT$^-$Ig$^-$ population consisted of relatively large cells (11–20 μm in diameter). The frequency, morphology and size distribution all support the notion that this population represents an early B lymphoid compartment.

Characterization of HIS50-binding cells in blood

Table 1 shows that about one-fifth of the peripheral blood mononuclear cells (PBMC) stained with HIS50. The vast majority of these were $\kappa^+\mu^+$ B lymphocytes. The others, not expressing κ or μ, were lymphoid in morphology. Part of the κ^+ or μ^+ cells were HIS50$^-$, only a subset of which were strongly $\kappa^+\mu^+$ plasma cells.

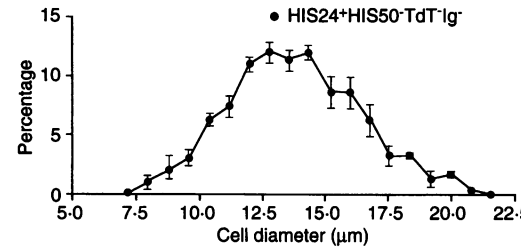

Figure 3. HIS24$^+$HIS50$^-$TdT$^-$Ig$^-$BM cell population consists of relatively large cells. Size distribution of HIS24$^+$HIS50$^-$TdT$^-$Ig$^-$ cells (mean$\pm$SD of 3 6-week-old animals; the diameter of 100 cells was measured).

Flow cytometric analysis of lymphoid tissues

Two colour flow cytometry analysis of blood and spleen further confirmed the predominant anti-B-cell reactivity of HIS50 (Fig. 4). As is shown in Table 2, one-third and two-thirds of the sIgM$^+$ lymphoid cells in blood and spleen, respectively, expressed the HIS50 determinant on the cell

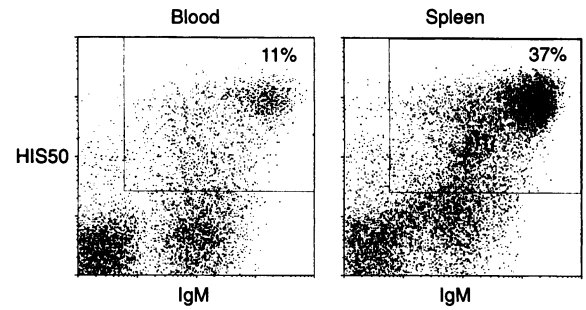

Figure 4. HIS50 reacts predominantly with B cells. Two-colour flow cytometry analysis of HIS50-Ag and IgM expression in blood and spleen. Cell suspensions from 3–4-month-old PVG rats were labelled with HIS50 (FITC) and 331 (anti-IgM (PE)) as described in Materials and Methods.

Table 2. Flow cytometry analysis of HIS50 expression in BM, blood and spleen

	Percentage labelled lymphoid cells in		
Cell subset	BM	Blood	Spleen
sIgM$^+$ sHIS50$^+$	14; 14*†	12; 11	32; 37; 27
sIgM$^+$ sHIS50$^-$	4; 3	23; 21	21; 19; 21
sIgM$^-$ sHIS50$^+$	32; 35	3; 3	4; 4; 3
sIgM$^-$ sHIS50$^-$	50; 48	62; 65	43; 40; 49

Cells from 14–16-week-old male PVG rats were stained with monoclonal anti-rat IgM (HIS40) conjugated to PE and HIS50 followed by anti mouse IgM conjugated to FITC. Examples of the flow cytometry profiles are shown in Fig. 4.

*Percentages are given from individual rats.

†Numbers represent percentages of cells with lymphoid forward and sideward scatter profiles.

surface. The apparent discrepancy in the percentage of HIS50$^+$ cells among IgM-expressing cells in blood between Table 1 and Table 2 (79 versus 34%) may be due to the difference in age of the rats analysed (5–6 weeks versus 3–4 months). With increasing age, a decreasing proportion of circulating B cells may have the immature phenotype IgM$^+$HIS50$^+$.[26] Figure 4 clearly shows that in blood and spleen levels of sHIS50 binding varied and that both sHIS50^{low} and sHIS50^{high} cells were present. Cells expressing relative high levels of sHIS50 were predominantly found among the sIgM^{high} B cells. Virtually all sIgM cells were also sHIS50^{high}. Lower levels of sHIS50 expression were predominantly found among sIgM^{low} cells; in particular in spleen, a significant fraction of the sIgM^{low} B cells was also sHIS50^{low}.

In BM, flow cytometric analysis showed that more than 75% of the sIgM$^+$ B cells expressed HIS50 determinant on the cell surface (Table 2). In this organ, many sIgM$^-$ cells were sHIS50$^+$, and were sHIS50^{high}. From the cytospin data it was evident that these cells were largely pro- and pre-B cells.

HIS50-binding to lymphoid tissues

In sections of spleen stained for HIS50 Ag by immunoperoxidase or immunofluorescence techniques, staining of HIS50 was largely confined to B-cell areas, i.e. lymphoid follicles and marginal zone (Fig. 5a). Marginal zone cells were strongly stained, while corona cells showed staining intensities varying from undetectable to strong. Germinal centre cells were weakly to moderately stained. In red pulp, solitary both strongly and weakly HIS50$^+$ cells were present which did not look like plasma cells. The outer periarteriolar lymphocyte sheath (PALS) contained rare scattered HIS50$^+$ cells. With regard to non-hemopoietic components, arterial vessels also bound HIS50. These data confirm flow cytometry analysis showing differential expression of HIS50 Ag among B-cell subsets.

In lymph nodes, HIS50 stained germinal centres, some scattered cells in the lymphocyte corona and rare cells in the paracortical areas (Fig. 5b). Scattered strongly HIS50$^+$ cells were in the medullary cords. Peijer's patches showed a staining pattern similar to that of lymph nodes, i.e. germinal centres, a few cells in the lymphocyte corona, virtually no cells in the T-cell areas and some scattered cells in the dome area. Thymus showed few scattered HIS50$^+$ lymphoid cells, in both cortex and medulla. These cells could be B lymphoid.[27] In addition septa and capsule were stained with HIS50.

HIS50 binding to sections of non-lymphoid organs

The binding of HIS50 to non-lymphoid organs was studied by immunoperoxidase staining of sections from a large variety of organs including liver, pancreas, kidney, intestine, ovary, heart and brain. Most tissues showed an overall diffuse staining. All epithelia showed HIS50 binding. The nuclei of a variety of cell types (including muscle, liver parenchyma, adrenal gland, and the islets of Langerhans) bound HIS50. In the pancreas the luminal side of the distal tubules and the collecting tubules, as well as the parietal layer of Bowman's capsule were stained. Epithelial expression of HIS50 Ag correlates well with the abundant expression of CD24 mRNA reported for epithelia in rat.[28,29] Also in man, CD24 mAb

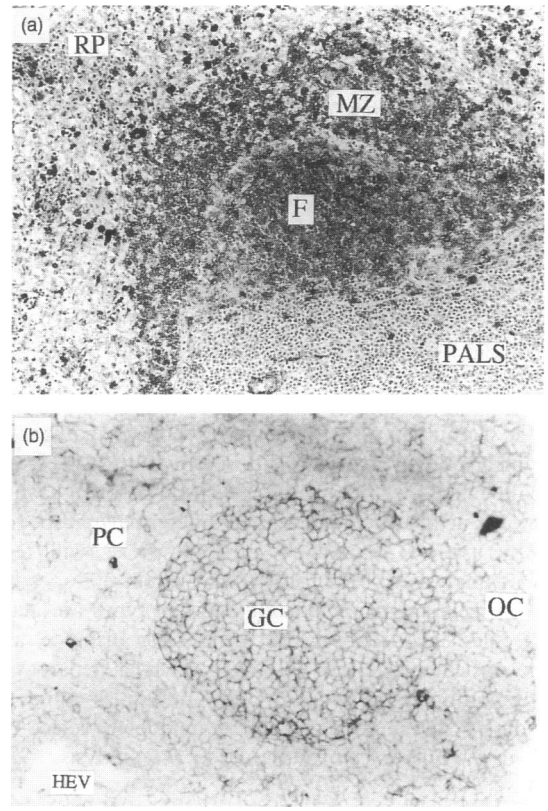

Figure 5. Differential expression of HIS50 Ag among B cell subsets. HIS50 staining (immunoperoxidase, see Materials and Methods) of frozen section of (a) spleen of a 14-week-old PVG rat (Obj. × 10) and (b) mesenterial lymph node. F, B cell follicle; MZ, marginal zone; PALS, periarteriolar lymphocyte sheath; RP, red pulp; GC, germinal centre; HEV, high endothelial venule; PC, para cortex; OC, outer cortex.

bind to a variety of epithelial cells[30] and carcinomas, and CD24 mRNA has been detected in various carcinomas.[29]

HIS50 binding to tissues of man

mAb to human CD24 have been reported to recognize marginal zone cells and follicular mantle cells strongly, but germinal centres weakly.[30] Such pattern of binding to peripheral B cell populations is unlike that of HIS50, and CD24 mRNA,[29] in rat. CD24 proteins may be differentially glycosylated by different cell types, and mAb may vary in their ability to recognize different glycoforms of CD24. In human lymph node and tonsil, HIS50 mAb recognized germinal centres rather than follicular mantles, and thus exhibited a pattern like that seen in rat. Assuming that HIS50 binding proteins in both rat and human germinal centres are encoded by CD24 genes, these observations suggest that HIS50 detects CD24 glycoforms in human tissue that are not recognized by the current panel of anti-human-CD24 mAb. In addition to specific germinal centre staining, weak granular binding of HIS50 to lymphoid cell nuclei was seen in the human tissues. Nuclear binding had been observed for HIS50 in rat for nuclei of a variety of non-lymphoid cells (see paragraph above), but has, to our knowledge, not been reported for mAb against human CD24. As reported earlier in the Results section, rat BM cells that

were HIS50$^+$ appeared to contain a large cytoplasmic pool of HIS50 Ag. In man, anti-human-CD24 mAb also detect a cytoplasmic pool of CD24 protein, in granulocytes.[30] Studies on blood and cell lines of human origin further indicated HIS50 epitope recognition intracellularly. Human peripheral blood mononuclear cells (PBMC) were all negative for HIS50 binding when viable, but exhibited binding to the cytoplasm after fixation. The same observations were made for B lineage lymphoma cell lines Raji and BJAB. Fixed T leukemia cell line MOLT4 bound HIS50 to the nucleus in a pattern excluding nucleoli; in addition, binding around the nucleus was observed. Fixed embryonic lung fibroblast cell line HEL152 also bound HIS50 to the nucleus except nucleoli, and showed additional dotty staining of what appeared to be the plasma membrane. MAb against human CD24 have been reported unable to bind to the surface of PBMC, Raji and BJAB, and MOLT4 to any significant extent;[31,32] binding after fixation may not have been attempted. Further understanding of differential epitope recognition by anti-CD24 mAb of various species will require molecular analysis to establish to what extent epitopes in the various subcellular compartments and cell types are actually on molecules encoded by CD24 genes. For instance, a hint that the nuclear binding of HIS50 to rat liver hepatocytes is not representing recognition of CD24 molecules comes from the study by Akashi *et al.* which is unable to detect CD24 mRNA in the liver by Northern and *in situ* analysis.[29]

DISCUSSION

The identification of HIS50 Ag, a rat homologue of CD24 and HSA, allowed us to define new compartments of B lymphoid cells in rat. In addition it provided us with information on the expression pattern of rat CD24 – as recognized by HIS50 – the function of which is still unknown.

In this paper we showed that, analogous to HSA and CD24, and predicted by the cDNA sequence at least part of the HIS50-binding molecules present on B lymphoid cells in BM were anchored to the cell membrane through a GPI–lipid anchor. The decrease by PI–PLC treatment in HIS50-staining was about 50%, consistently less than for Thy-1. One explanation is that part of the anchors cannot be cleaved by the PI–PLC. Another explanation is that HIS50-binding molecules can be expressed as both integral transmembrane and GPI-anchored proteins. Support for this last possibility is that one of the HSA genes which have been isolated so far could encode a form of HSA with a transmembrane domain and a cytoplasmic tail.[33]

Among lymphoid cells of rat, HIS50-Ag expression was virtually restricted to B lymphoid cells as is CD24 expression in man. This is in contrast to HSA expression in mouse in which the vast majority of thymocytes and BM non-lymphoid cells is HSA$^+$.[34,35] Outside the B lymphoid lineage in BM, HIS50 Ag was expressed by a very small number of myeloid cells with doughnut-shaped nuclei and granules. In man, a proportion of the granulocytic lineage expresses CD24 Ag.[31]

Within the B lineage in BM, HIS50 Ag was expressed by the vast majority of B lymphoid cells. If we assume that the antigens studied are continuously expressed during differentiation and proliferation (i.e. they do not disappear temporarily), we can deduce the following model of B lymphocyte development in the rat. Pluripotent haemopoietic stem cells are Thy-1^{high}LCA$^-$; about 0·3% of nucleated BM cells have this phenotype.[2] Then the cells start expressing a B cell form of LCA (HIS24) giving the newly defined Thy-1$^+$HIS24$^+$HIS50$^-$TdT$^-$(c+s)μ^- population constituting 1·6% of BM nucleated cells. Figure 3 gives the size distribution of this BM B lymphoid cell population. The median diameter is in between 13 and 14 μm, while the median diameter of all HIS24 expressing cells in BM of 6-week-old rats is 9 μm.[22] In general, large HIS24$^+$ cells are cycling cells.[22] Large cells are abundant in the early compartments and gradually decrease in relative numbers as the cells mature.[22] As shown here, most of the Thy-1$^+$HIS24$^+$HIS50$^-$TdT$^-$(c+s)μ^- cells are large, the largest BM B lymphoid population described in rat so far. Because of this, they may well represent a rapidly dividing early precursor compartment.

Since all TdT$^+$ cells are HIS24$^+$,[3] TdT appears to be expressed simultaneously with or after HIS24. The timing of TdT expression in relation to HIS50 Ag is not clear since TdT expressing cells have a variety of phenotypes. A small part of the TdT$^+$ cells do not express HIS50 Ag while the large majority of TdT$^+$ cells expresses HIS50 Ag. In addition, some TdT$^+$ cells express cytoplasmic μ chains.[3,22] If TdT would be expressed from the onset of HIS50 Ag expression until the onset of cμ expression the percentage of TdT$^+$ cells in BM would be higher than it actually is. One explanation is that not all developing B lymphoid cells go through a immunohistologically detectable TdT-positive stage. Another possibility is that the timing of TdT expression within the B lineage varies from cell to cell. Alternatively, the LCA$^+$HIS50$^+$TdT$^-$(c+s)μ^- population may belong to a separate lineage. The Thy-1$^+$LCA$^+$HIS50$^+$cμ^- stage is followed by the Thy-1$^+$LCA$^+$HIS50$^+$cμ^+ stage, whereafter in addition L chains are expressed to give rise to newly formed B cells. Rat BM cell development *in vitro* appears to exhibit the same sequence of gene expression.[36]

The timing of expression of HIS50 Ag seems similar to the expression of HSA in mouse B lymphocyte development, namely after LCA and before cμ-expression.[37] The HIS24$^+$HIS50$^-$TdT$^-$Ig$^-$ population in rat is phenotypically the closest to the B220$^+$HSA$^-$CD43$^+$BP1$^-$ population in mouse in which TdT expression is low (Fraction A, 37). In Fractions B and C (B220$^+$CD43$^+$HSA$^+$BP1$^{-/+}$) TdT expression is high.[37] Similarly, in rat BM all TdT$^+$ cells are HIS24$^+$ and most TdT$^+$ cells are HIS50.$^+$ Also in man the vast majority, but not all, of TdT$^+$ cells express CD24.[38] All cμ^+ cells and almost all sμ^+ cells in rat BM express HIS50 Ag, analogous to CD24 and HSA expression on cμ^+ and sμ^+ cells in man and mouse, respectively.[39,40]

Peripheral B cells showed differential expression of HIS50 Ag. Virtually all IgM^{bright} cells in spleen and blood also expressed high levels of HIS50 Ag, while the majority of IgM^{dull} cells expressed low to undetectable levels of this Ag. As we have shown previously, IgM^{bright} cells comprise both B cells newly generated in the BM (immature B cells) and marginal zone B cells, and IgM^{dull} cells largely represent recirculating follicular B cells.[26] Thus, our data presented here indicate that both immature B cells and splenic marginal zone B cells all express high levels HIS50 Ag, while follicular B cells express variable levels (ranging from undetectable to high) HIS50 Ag. Immunohistological staining of spleen confirmed the high expression levels of HIS50 Ag on marginal zone B

cells and the heterogeneous expression on follicular B lymphocytes. Furthermore, tissue section analysis revealed that germinal centre B cells express HIS50 Ag. The mRNA of CD24 has also been detected in rat lymph node (LN) germinal centres.[29] Similarly, also in other species (man, mouse), B cell populations differ in their levels of HSA, albeit that in these species all B cells express at least some HSA.[39,41–43] Despite some differences between various antibodies used, the same general pattern is observed in which follicular B cells express the lowest levels of HSA, and marginal zone B cells, immature B cells and activated B cells the highest levels.[42,43]

Within the B lineage, the abundance of CD24 molecules in immature cells, and in subsets of mature cells in at least three species suggests a common role of these molecules in the development and function of B lymphoid cells. In chimeric mice deficient for HSA, B cells do mature.[44] However, an altered degree of bone marrow and blood chimerism may suggest that HSA expression influences the maturation of B cells. Using pre-B cell clones deficient for HSA it was shown that the expression of HSA on pre-B cells modifies the binding specificity of very late antigen (VLA)-4 for vascular cell adhesion molecule-1 (VCAM-1) and fibronectin.[45] In this way regulation of HSA expression during cell maturation could be a mechanism for associating cells of various maturation stages with specific developmental compartments.

Anti-CD24 mAb recognize the vast majority of mouse thymocytes,[46] but only minor subsets of thymocytes in man and rat. However, in young rats, abundant CD24 mRNA is present in the thymus.[28] Therefore it seems likely that HIS50 mab recognizes only a subset of rat CD24.

Recently, the function of HSA in T-cell development was addressed by generation of transgenic mice constitutively expressing HSA throughout all stages of T-cell maturation (normally HSA is down-regulated during thymocyte development). The continuous expression of HSA resulted in a diverse phenotype. While Hough *et al.*[47] reported severely impaired thymocytopoiesis, Nielsen *et al.*[48] saw little effect on the thymus, but reported an augmented secondary Ab response. In addition, Ab blocking studies and gene transfer experiments have indicated that one of the functions of HSA is the co-stimulation for $CD4^+$ T-cell growth.[11–13]

The elucidation of the exact functions of HSA/CD24/HIS50 Ag at the various stages of lymphoid and other haemopoietic cell development will help at understanding underlying mechanisms involved in their generation and function.

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