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THY1 ANTIGEN AND B LYMPHOCYTE DIFFERENTIATION IN THE RAT[1]

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Lymphocytes that bear both sIg and Thy1 antigen are among the least mature members of the B lymphocyte series in the rat. Evidence supporting this conclusion was derived in part from a study of the distribution and time course of appearance of sIg$^+$, Thy1$^+$ cells in normal and regenerating lymphoid tissues. The highest percentage of B lymphocytes that express Thy1 in adult rats was present in bone marrow (76%), the lowest in lymph node (<10%), with spleen being intermediate (26%). There was a marked age-related decrease in the percentage of splenic B cells that expressed Thy1, ranging from 94% at birth to 15% in rats greater than 12 weeks of age. Similarly, B cells in peripheral lymphoid tissues of sublethally irradiated rats showed a disproportionately high percentage of Thy1$^+$ B cells early in the course of regeneration, followed by a reversion to normal proportions of Thy1$^+$ and Thy1$^-$ B cells. This reversion to normal did not occur in thymectomized, irradiated, and bone marrow reconstituted (TXBM) rats. In other experiments, between 78% and 93% of complement receptor lymphocytes (CRL) and essentially all direct and indirect plaque-forming cells directed to SRBC or TNP were Thy1$^-$. Lastly, the anatomical localization of sIg$^+$, Thy1$^+$ cells within spleen and lymph node was determined on frozen sections by dual immunofluorescence. Thy1$^+$ B cells were restricted to primary lymphoid follicles and to the lymphocyte corona of secondary lymphoid follicles. Thy1 was not seen on sIg$^+$ cells in germinal centers, marginal zones, or on plasma cells. These results, in their aggregate, suggest that sIg$^+$, Thy1$^+$ cells may belong to the antigen independent and/or thymus-independent phase of B lymphocyte development.

Thy1,[4] previously called θ, was first described by Reif and Allen (1) as an allelically expressed antigen present on thymocytes and in nervous tissues of mice. Subsequently shown to be restricted within the lymphoid cell series to thymocytes and thymus-dependent lymphocytes in the mouse (2), Thy1 has been used extensively as a general T cell marker in this species. The Thy1.1 allele, indistinguishable by absorption analysis from that in the mouse, was found to be expressed on rat thymocytes (3). Unlike the mouse, however, most rat peripheral T cells were found to be Thy1$^-$ (3, 4). The different distribution of Thy1 in the rat was underlined by the finding of Williams (5) and of Hunt *et al.* (6) that approximately two-thirds of sIg^{+4} lymphocytes in young rat bone marrow were Thy1$^+$. In an earlier study from this laboratory, Ritter *et al.* (7) reported the presence of Thy1 on a subset of sIg$^+$ cells in rat spleen and lymph node as well as on all sIg$^+$ cells in adult rat bone marrow. Again, in contrast to the mouse, Goldschneider *et al.* (8) have recently demonstrated that pluripotent hemopoietic stem cells and myeloid progenitor cells in the rat are strongly Thy1$^+$, as are putative thymocyte progenitors (9). The presence of Thy1 on these cell types has enabled them to be isolated on the fluorescence-activated cell sorter.[5]

The purpose of this study is to further characterize the population of B lymphocytes in the rat that expresses the Thy1 antigen. Using dual immunofluorescence for sIg and Thy1, we have determined that Thy1 is present on an immature population of B lymphocytes in bone marrow and lymphoid follicles, and that it is absent from antibody-forming cells and from most complement (C) receptor lymphocytes. This distribution appears to be unique among B cell markers, all others of which are at least partially represented on mature B cells (2, 10–14). Consequently, it is likely that Thy1 will prove to be a useful probe with which to study the early stages of B lymphocyte development in the rat.

MATERIALS AND METHODS

Animals and tissues. (Lewis × DA)F_1 hybrid rats of both sexes were used in all experiments. Breeding stocks of these animals were maintained at the University of Connecticut Health Center. Bone marrow, cervical lymph node, and spleen cells were collected as previously described (7). Cell viability was checked with 0.1% trypan blue.

Lymphocyte-rich fractions were prepared from suspensions of spleen cells by isopycnic centrifugation on a Ficoll-Hypaque gradient (15). The recovery of viable nucleated cells in bone marrow and lymph node after irradiation with 675 R was determined by comparing the number of cells obtained from

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[4] Abbreviations: Thy1$^+$, cell bearing Thy1 antigen; sIg$^+$, cell bearing surface immunoglobulin; CRL, complement receptor lymphocyte; EAC, erythrocyte-antibody-complement complex; TXBM, neonatally thymectomized, gamma irradiated, bone marrow reconstituted; FITC, fluorescein isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate; EDTA, ethylenediaminetetraacetic acid; i.m., intramuscular; s.c., subcutaneous; D-PBS, Dulbecco's phosphate-buffered saline.
[5] Goldschneider, I., *et al.* (1979) Analysis of rat hemopoietic cells on the fluorescence-activated cell sorter. Submitted for publication.

one femur and one tibia and the same superficial and deep cervical lymph nodes from irradiated and nonirradiated rats.

Irradiation. Whole body irradiation was delivered from dual ^{137}Cs sources (128 rads per minute) in a Gammacell 40 irradiator (Atomic Energy of Canada, Ltd.). Irradiated and control (nonirradiated) rats were maintained on oxytetracycline (Pfizer, N. Y.) 1.0 mg/ml in their drinking water for the duration of the experiment.

Thymectomy. Neonatally thymectomized rats were the generous gift of Dr. R. W. Barton. At 3 to 4 weeks of age they were irradiated with 800 R and injected with 1×10^8 nucleated syngeneic bone marrow cells i.v. The TXBM[4] and untreated control rats were maintained on oxytetracycline (Pfizer), 1.0 mg/ml in their drinking water.

Antisera. Fluorescein isothiocyanate- (FITC) conjugated $F(ab')_2$ fragment of the 7S fraction of rabbit antiserum to purified rat brain Thy1 antigen was the generous gift of Dr. R. J. Morris. This antiserum was used at a concentration of 70 μg/ml. The preparation and absorption of this antiserum is described in full elsewhere (16).

Mouse alloantiserum against Thy 1.1 (C3H anti-AKR/J thymocyte serum) was prepared according to the protocol of Reif and Allen (1). The resultant serum was used as either a whole serum or an immunoglobulin-enriched fraction after precipitation with 50% saturated ammonium sulphate.

IgG fraction of FITC-conjugated rabbit anti-rat IgG (heavy and light chains), tetramethyl rhodamine isothiocyanate- (TRITC) conjugated $F(ab')_2$ fraction of rabbit anti-rat IgG (heavy and light chains), TRITC-conjugated IgG fraction of goat anti-mouse IgG (heavy and light chains), and rabbit anti-rat γ-globulin were purchased from Cappel Laboratories (Downington, Pa.).

Immunofluorescence of cell suspensions

Dual immunofluorescence for sIg and Thy1. 1 to 2×10^6 cells were incubated for 20 min at 4°C, sequentially with 10 μl of FITC rabbit anti-rat IgG and mouse anti-Thy1.1, followed by TRITC goat anti-mouse IgG. The cells were washed twice with PBS + 0.02 M sodium azide before addition of each successive antiserum. Dual immunofluorescence with FITC $F(ab')_2$ rabbit anti-rat brain Thy1 serum and TRITC $F(ab')_2$ rabbit anti-rat IgG was carried out by the simultaneous incubation of 1×10^6 cells with 10 μl of both conjugated antisera for 20 min at 4°C.

Specificity controls. The specificity of the rabbit anti-rat brain Thy1 serum has been demonstrated elsewhere (7, 16). In the present experiments, aliquots of this antiserum absorbed with an equal volume of rat thymocytes were routinely included as negative controls. Moreover, in single and dual immunofluorescence experiments (data not shown) it was demonstrated that the mouse anti-Thy1.1 alloantiserum stained the same population of spleen cells as did the rabbit anti-rat brain Thy1 heteroantiserum. Normal serum controls were routinely included in indirect immunofluorescence experiments. In dual immunofluorescence experiments, the percentage of cells that stained with one conjugate was shown to be similar in the presence or absence of the second conjugate. For dual immunofluorescence with the use of mouse alloantiserum to Thy1.1, the FITC rabbit anti-rat IgG was passed through a normal mouse serum Sepharose 4B affinity column (mouse serum coupled to CNBr-activated Sepharose 4B) (Pharmacia Fine Chemicals, N. J.) to remove cross-reacting antibodies to mouse immunoglobulins. Similarly, TRITC goat anti-mouse IgG was passed through a normal rat serum Sepharose 4B affinity column to remove cross-reacting antibodies to rat immunoglobulins. All antisera were deaggregated by ultracentrifugation before use.

Immunofluorescence of frozen sections. Frozen sections of spleen and lymph node were first overlaid with TRITC $F(ab')_2$ rabbit anti-rat IgG, washed for 5 min in cold Dulbecco's phosphate-buffered saline (D-PBS), and then overlaid with FITC $F(ab')_2$ rabbit anti-Thy1 and washed twice for 5 min in cold D-PBS. Controls consisted of sections similarly treated with TRITC $F(ab')_2$ rabbit anti-rat IgG passed through a Sepharose 4B-linked rat IgG affinity column, and thymocyte absorbed FITC $F(ab')_2$ rabbit anti-Thy1.

Optical system for fluorescence. Cell suspensions were examined at 400× magnification by using a Zeiss RA microscope equipped for U.V. epi-illumination with a 50-watt mercury vapor lamp and filter combinations for both fluorescein and rhodamine. Total nucleated cell counts were made under dark-field illumination. Photomicrographs were taken with Kodak Tri-X film at magnifications of 320× for cell suspensions and 128× for tissue sections. Printing of 35mm negatives involved a further enlargement of approximately 4.6×.

Assay for C receptor lymphocytes (CRL)

Preparation of erythrocyte-antibody-complement complexes (EAC). Sheep red blood cells (SRBC) (Scott Laboratories, Fiskeville, R. I.) were washed three times in D-PBS and then incubated with a volume of a 1:200 dilution of hemolysin (Cordis Laboratories, Miami, Fla.) to make a 5% (v/v) solution, for 30 min at 37°C. The SRBC were then washed three times in D-PBS and resuspended in D-PBS as a 5% (v/v) solution. An equal volume of a 1:3 dilution of fresh AKR/J mouse serum was then added as a C source, and the suspension was incubated for 30 min at 37°C. The resulting EAC were washed three times in D-PBS + 0.01 M EDTA[4] + 0.02 M sodium azide for use in the complement receptor assay. The sodium azide was added to prevent formation of Fc rosettes (17); the EDTA to inhibit the formation of EAC rosettes by macrophages and granulocytes (18). It was also established that EAC rosettes were not formed in the absence of C (data not shown).

Rosette assay for CRL. One hundred microliters of a spleen cell suspension at 8×10^6/ml in D-PBS + 0.01 M EDTA + 0.02 M sodium azide were incubated in a round-bottomed microtiter plate (Cooke Engineering, Alexandria, Va.) with an equal volume of a 5% solution of EAC for 50 min at 37°C. An equal volume of 1% crystal violet in D-PBS was added to the CRL suspension before counting the rosettes. This stain facilitated detection of nucleated cells in rosettes and counting of nonrosetting nucleated cells in the background of SRBC.

Combined immunofluorescence and CRL assay. In some experiments, spleen cells were labeled with fluorescent antisera before rosetting. Preliminary experiments showed that the fluorescent labeling did not affect the number of rosettes formed (data not shown). Rosettes were examined by fluorescence microscopy by applying a single drop of the rosette suspension to an alcohol-cleaned slide and applying a coverslip. The rosettes were made planar by allowing them to stand for 1 hr at 4°C under the coverslip, thereby clearly exposing the outline of the central nucleated cell.

Assay for hemolytic plaque-forming cells (PFC)

Trinitrophenylation of SRBC. The method of Rittenberg and Pratt (19) was used to conjugate 2,4,6 trinitrophenyl (TNP) to SRBC, with the exception that D-PBS was used in place of modified barbital buffer. Trinitrophenylation of bovine serum albumin (BSA) was carried out as described (20). The hapten-

to-protein molar ratio was calculated to be 20.

Immunization with SRBC or TNP. Rats were primed with 5×10^8 SRBC i.v. or i.p. and boosted 9 or 10 days later with 1×10^8 SRBC i.v. or i.p., respectively. Rats were primed with 2.5 mgs TNP-BSA in complete Freund's adjuvant (Difco) intramuscularly (i.m.) and subcutaneously (s.c.) (2 parts i.m. and 1 part s.c.), and boosted with 1.0 mg TNP-BSA in PBS i.m. and s.c. 9 or 10 days later. Spleen and lymph node cells were harvested 8 to 9 days after the primary injection or 3 to 4 days after the booster injection.

Assay for PFC. The assay for PFC in liquid medium was based on the method of Cunningham (21). To *400 μl* of the spleen or lymph node cell suspension in MEM or RPMI was added 50 μl of guinea pig serum as a source of C and 50 μl of a 5% (v/v) solution of ×3 washed SRBC or TNP-SRBC. After brief vortexing to ensure mixing, 25 μl of this suspension were applied to a microscope slide between two strips of Scotch tape placed 1.5 cm apart. A coverslip was applied and sealed with nail varnish. The slide was then incubated for 50 min at 37°C. Based on preliminary determinations, a concentration of spleen or lymph node cells was used that resulted in the presence of a single nucleated cell within most plaques.

In assays for indirect PFC, rabbit anti-rat γ-globulin at a final concentration of 1:200 or 1:400 was added to the incubation mixture. Before this, the spleen or lymph node cell suspension was diluted to prevent the formation of direct plaques. For example, if the mean number of direct plaques per slide was previously determined to be 10, a 10-fold dilution of the spleen or lymph node cells was made. This was possible because the numbers of indirect plaques exceeded direct plaques in all experiments.

Combined immunofluorescence and PFC assay. In some experiments, spleen or lymph node cells were labeled with FITC $F(ab')_2$ rabbit anti-Thy1 before conducting the PFC assay. Preliminary experiments showed that fluorescent labeling did not affect the number of direct or indirect plaques formed (data not shown). Only plaques that contained red cell ghosts and one centrally located nucleated cell were counted.

RESULTS

Distribution of sIg^+, $Thy1^+$ lymphocytes. The development of sIg^+ cells in rat bone marrow, spleen, and lymph node was studied by dual immunofluorescence by using antisera to rat IgG and Thy1 antigens (Fig. 1). Results in Table I show that approximately 76% of sIg^+ cells in adult rat bone marrow were $Thy1^+$, but that only 26% of sIg^+ spleen cells and 8% of sIg^+ lymph node cells were $Thy1^+$. In contrast, only about 25% of $Thy1^+$ cells in bone marrow and lymph node and about 58% in spleen were sIg^+.

Marked age-related differences in the percent of sIg^+, $Thy1^+$ cells were observed in spleen. As shown in Table II, the proportion of sIg^+ spleen cells that were also $Thy1^+$ steadily decreased from a maximum of 94% at birth to a minimum of 15% by 12 weeks of age. This decline in sIg^+, $Thy1^+$ cells was not accompanied by a proportionate change in the percentage of total sIg^+ cells with age.

The results in Table II also show that the percentage of total $Thy1^+$ spleen cells declines as a function of age, from a high of 62% at birth to a low of 7% by 12 weeks. Yet, with the exception of the neonatal period, the percentage of $Thy1^+$ cells that bear sIg remains relatively constant (53 to 74%). This suggests that proportionate decreases in $Thy1^+$ cells occur among both the sIg^+ and sIg^- populations. In the neonatal spleen, a disproportionately high percentage of $Thy1^+$ cells are sIg^-, presumably reflecting the major involvement of the spleen in extramedullary hemopoiesis at this time (22). In this respect, the distribution of $Thy1^+$ cells among sIg^+ and sIg^- populations is similar in neonatal spleen and adult bone marrow (compare Tables I and II).

Location of sIg^+, $Thy1^+$ cells in spleen and lymph node. Frozen sections of lymph node and spleen were treated with TRITC $F(ab')_2$ rabbit anti-rat IgG and FITC $F(ab')_2$ rabbit anti-Thy1 and examined by dual immunofluorescence for differential staining. In lymph node, anti-IgG stained germinal center cells, follicular lymphocytes, and plasma cells, but only scattered cells in paracortex (Fig. 2*a*). Lymphocytes in primary follicles and in the corona of secondary follicles were also strongly $Thy1^+$ (Fig. 2*b*); but germinal center cells and plasma cells were $Thy1^-$. The vast majority of lymphocytes in the paracortex appeared to be sIg^-, $Thy1^-$. However, collections of lymphocytes in the immediate vicinity of post-capillary venules contained many sIg^-, $Thy1^+$ lymphocytes.

Dual immunofluorescence on spleen sections (Fig. 3) showed that many cells in periarteriolar lymphatic sheath stained with anti-Thy1 but not with anti-IgG. Conversely, many cells in the marginal zones and germinal centers stained strongly with anti-IgG but failed to show detectable fluorescence with anti-Thy1. As in lymph node, the primary follicles and lymphocyte coronas of secondary follicles in spleen stained with both anti-Thy1 and anti-IgG reagents. Scattered Ig^-, $Thy1^+$ and Ig^+, $Thy1^-$ cells were seen in red pulp of spleen. Many of the latter cells were plasma cells.

Regeneration of sIg^+, $Thy1^+$ lymphocytes in gamma-irradiated rats. The pattern of B cell regeneration in rats that had received 675 R whole body irradiation is shown in Table III. This dose reduced the nucleated cell population in bone marrow, spleen, and lymph node to 2.6%, 1.5%, and 1.5%, respectively, of control values at 9 days after irradiation. The number of sIg^+ cells in each of these tissues increased progressively after a variable lag period that was shorter in bone marrow compared with spleen and lymph node. By 42 days post-irradiation, the percent recovery of sIg^+ cells in bone marrow, spleen, and lymph node was 71%, 68%, and 58%, respectively.

Figure 4 shows the pattern of regeneration of B lymphocytes in irradiated rats as studied by dual immunofluorescence for sIg and Thy1 antigens. sIg^+, $Thy1^+$ cells were not detected in bone marrow 9 days after irradiation. However, normal percentages of sIg^+, $Thy1^+$ B cells were attained in this tissue by day 14 and remained constant thereafter. In contrast, the proportion of B cells that expressed Thy1 in spleen and lymph node of rats that had been irradiated 14 to 28 days previously was approximately 2 to 3 times greater than in normal animals. At 42 days after irradiation, levels of $Thy1^+$ B cells in spleen and lymph node approached those in nonirradiated control animals.

Distribution of sIg^+, $Thy1^+$ cells in TXBM rats. Suspensions of cells from 12-week-old TXBM rats were examined by dual immunofluorescence for sIg and Thy1 antigens. The results in Table IV show that the percentage of sIg^+ and $Thy1^+$ cells in bone marrow was similar in TXBM rats compared with normal, age-matched control animals. In spleen and lymph node, however, the percentage of both sIg^+ and $Thy1^+$ cells was increased approximately 2-fold in TXBM rats compared with normal animals. The increase in $Thy1^+$ cells in these tissues can be accounted for almost entirely by an increase in the ratio of $Thy1^+$:$Thy1^-$ B cells, as opposed to a selective loss of $Thy1^+$ sIg^- cells.

C receptor lymphocytes (CRL). The ontogeny and tissue distribution of CRL in the rat is shown in Figure 5. CRL are

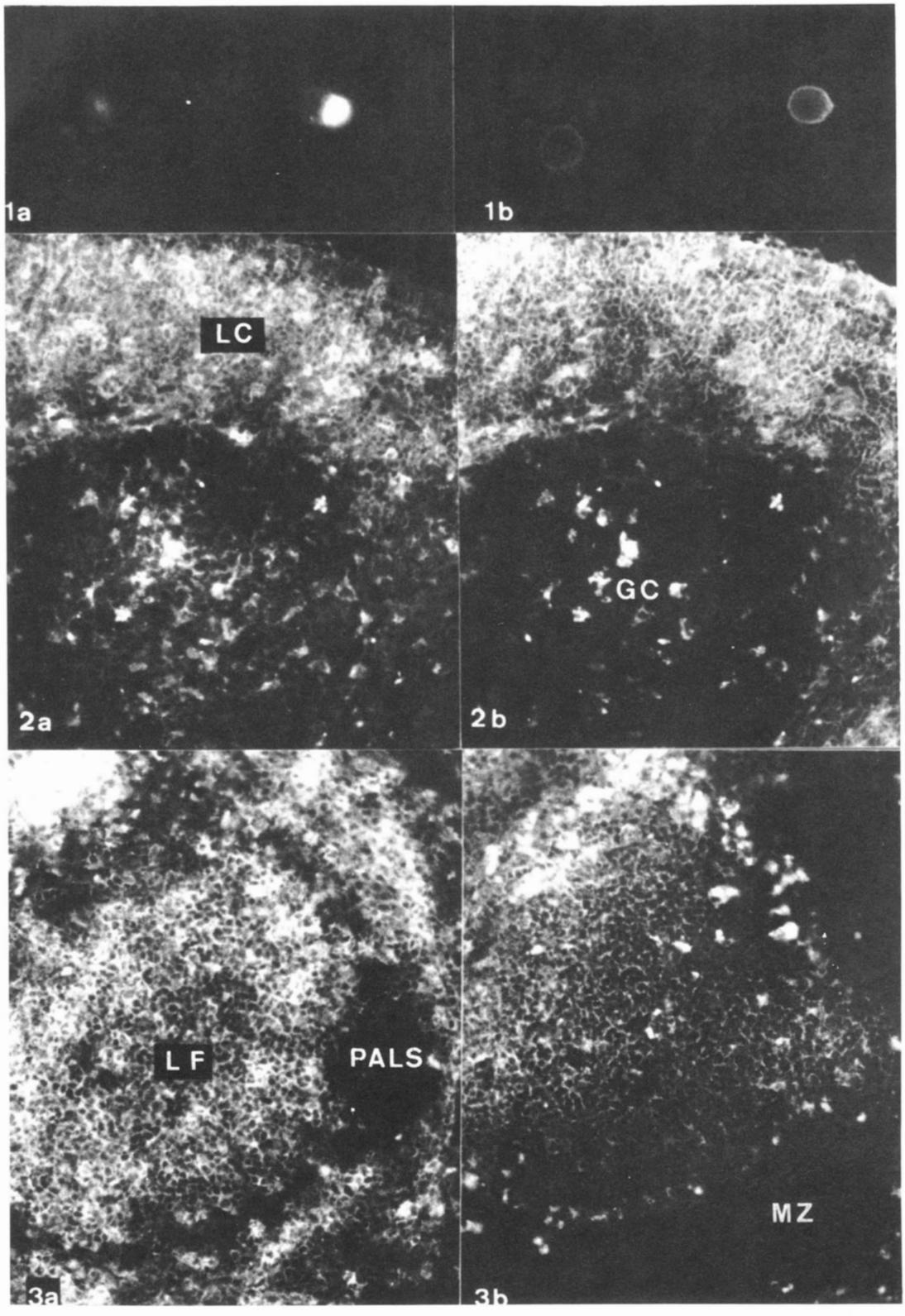

Figure 1. Dual immunofluorescence on spleen cells treated with anti-IgG and anti-Thy1. *a*, Spleen cells treated with TRITC $F(ab')_2$ rabbit anti-rat IgG under capping conditions (37°C for 15 min without sodium azide); *b*, followed by FITC $F(ab')_2$ rabbit anti-Thy1 under noncapping conditions (4°C for 20 min with sodium azide). Note dual staining of cell on right and independent capping of sIg and Thy1. sIg^-, $Thy1^+$ cell is present on left side of frame.

Figure 2. Dual immunofluorescence on a frozen section of rat lymph node treated with *a*, TRITC $F(ab')_2$ rabbit anti-rat IgG. Cells in both the germinal center (GC) and lymphocyte corona (LC) of the lymphoid follicle are positive. *b*, FITC $F(ab')_2$ rabbit anti-Thy1. Only cells in the lymphocyte corona are positive.

Figure 3. Dual immunofluorescence on a frozen section of rat spleen treated with *a*, TRITC $F(ab')_2$ rabbit anti-rat IgG. Cells in the lymphoid follicle (LF) and marginal zone (MZ) are positive; cells in the periarteriolar lymphoid sheath (PALS) are negative. *b*, FITC $F(ab')_2$ rabbit anti-Thy1. Only cells in the LF and PALS are positive.

uncommon in bone marrow of rats from birth through 10 weeks of age (maximum 2.7% of nucleated cells). CRL are also uncommon (≈5%) in spleen and lymph node from 3-week-old animals. Adult levels of CRL are reached in spleen (23.5%) and lymph node (14.4%) by 7 weeks of age.

The presence of sIg or Thy1 on CRL in spleen and lymph node from 6- to 8-week-old rats was studied by combined immunofluorescence and rosetting assay. Results in Table V show that more than 97% of CRL in these tissues are sIg^+. However, only a small minority of CRL in spleen (≈7%) and lymph node (≈22%) bear Thy1 antigen.

Plaque-forming cells (PFC). In these experiments, spleen or lymph node cells from rats immunized with either SRBC or TNP-BSA were incubated with FITC $F(ab')_2$ rabbit anti-Thy1 before being assayed for antibody secretion in the Cunningham (21) plaque-forming assay. Single antibody-secreting cells in the center of plaques were examined by fluorescence microscopy for the presence of Thy1 antigen. The data in Table VI show that cells secreting antibody to SRBC or TNP do not bear detectable Thy1. This includes both direct PFC (putative IgM-secreting cells) and indirect PFC (putative IgG-secreting cells).

Two observations help confirm the validity of the method. $Thy1^+$ nonantibody-secreting cells could be clearly observed in the background lawn of SRBC, demonstrating that Thy1 had not been cleared from the membrane of at least these cells during the incubation at 37°C. In addition, incubation of spleen cells for 50 min at 37°C after staining with rabbit anti-Thy1 did not affect the percent of $Thy1^+$ cells detectable by fluorescence (data not shown).

TABLE I

Distribution of sIg^+, $Thy1^+$ cells in adult rat tissues[a]

Tissue	% Positive Cells (±S.E.)			
	$sIg^{b,d}$	$Thy1^{c,d}$	sIg, Thy1/ Total sIg	sIg, Thy1/ Total Thy1
Bone marrow	8.4 ± 0.5	28.6 ± 2.5	76.3 ± 3.2	23.1 ± 3.2
Spleen	40.2 ± 1.9	16.8 ± 1.2	25.8 ± 2.3	57.6 ± 4.5
Lymph node	26.4 ± 1.6	8.1 ± 0.7	7.5 ± 1.1	25.3 ± 3.6

[a] Six to eight weeks of age.
[b] sIg detected by TRITC $F(ab')_2$ rabbit anti-rat IgG.
[c] Expressed as a percentage of total nucleated cells.
[d] Thy1 detected by FITC $F(ab')_2$ rabbit anti-rat Thy1.

TABLE II

Age-related changes in sIg^+ and $Thy1^+$ cells in rat spleen

Age	% Positive Cells (±S.E.)			
	$sIg^{a,b}$	$Thy1^{b,c}$	Thy1, sIg/ Total sIg	Thy1, sIg/ Total Thy1
Neonate[d]	15 ± 4	62 ± 4	94 ± 5	22 ± 5
3 weeks	34 ± 2	34 ± 3	76 ± 8	74 ± 2
5–8 weeks	34 ± 2	24 ± 4	52 ± 11	67 ± 7
>12 weeks[e]	21 ± 4	7 ± 1	15 ± 1	53 ± 8

[a] sIg detected by FITC rabbit anti-rat IgG.
[b] Expressed as a percentage of total nucleated cells in lymphocyte-rich fractions.
[c] Thy1 detected by C3H anti-AKR/J thymocyte serum developed by TRITC goat anti-mouse IgG.
[d] Neonate, within 24 hr of birth.
[e] Range, 12 weeks to 2 yr.

DISCUSSION

The results of the present study strongly indicate that sIg^+ cells that bear Thy1 antigen are among the least mature members of the B lymphocyte series in the rat. We have found, for example, that the distribution of sIg^+, $Thy1^+$ cells in lymphohemopoietic tissues correlates with the distribution of functionally immature B cells, both populations being highest in bone marrow (23, 24), intermediate in spleen (25, 26), and lowest in lymph node (27). Similarly, the pattern of regeneration of sIg^+, $Thy1^+$ cells after sublethal irradiation of adult rats suggests the

TABLE III

Mean percent regeneration of sIg^+ cells in adult rats after 675 R whole body gamma-irradiation

Days after Irradiation	Regeneration[a]					
	Bone marrow		Spleen		Lymph node	
	Total[b] cells	sIg^c	Total cells	sIg	Total cells	sIg
	%					
9	2.6	1.3	1.5	0.4	1.5	0.7
14	14.2	10.0	3.1	1.9	2.2	1.1
28	100.0	75.0	48.8	47.8	45.6	35.6
42	79.0	70.9	62.5	68.3	65.0	58.3

[a] Compared to values in unirradiated age-matched controls (See *Materials and Methods*).
[b] Total nucleated cells.
[c] Detected by TRITC $F(ab')_2$ rabbit anti-rat IgG.

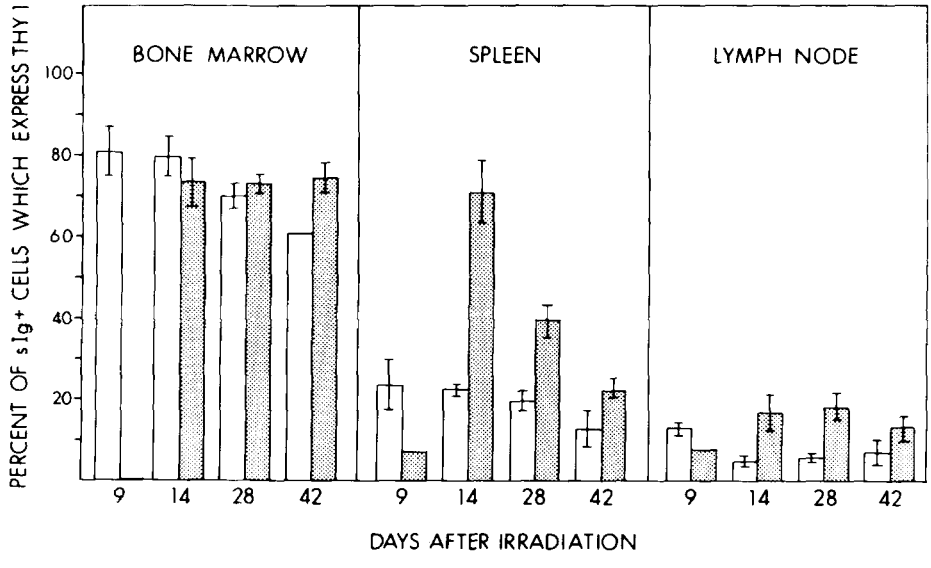

Figure 4. The effect of sublethal irradiation on the percentage of sIg^+ cells which express Thy1 in adult rat bone marrow, spleen, and lymph nodes. ▨, irradiated with 675 R; □, unirradiated, age-matched controls. Each bar represents the percent of sIg^+, $Thy1^+/sIg^+$ cells (mean + S.E.).

recolonization of peripheral lymphoid tissues with immature B cells. As in normal ontogeny (28), regenerating sIg^+ cells appeared early in bone marrow and slightly later in peripheral lymphoid tissues. Moreover, several weeks after irradiation, the ratio of $Thy1^+$:$Thy1^-$ B cells in spleen and lymph node was 2 to 3 times above normal, returning towards normal shortly thereafter. This is reminiscent of the age-related decrease in the ratio of $Thy1^+$:$Thy1^-$ B cells that occurs in spleen of normal rats after birth. These findings are consistent with the results of Yang *et al.* (29) and Strober (25) that suggested that many B lymphocytes are released from bone marrow in a relatively immature state and undergo further differentiation in spleen before they become fully functional (see References 26 and 30 for discussion).

In a previous report from this laboratory (7), higher percentages of sIg^+ cells in bone marrow (100%), spleen (90%), and lymph node (~50%) were found to express Thy1 than in the present study. Conversely the percentage of total sIg^+ cells in each of these tissues was lower than reported here, even allowing for the inclusion of RBC in the former cell counts. The most likely explanation for this apparent discrepancy is a difference in the sensitivity of the immunofluorescence procedures, alloantiserum to rat Ig light chains being less sensitive than heteroantiserum to rat IgG. It is important to note, therefore, that Williams (5) and Hunt *et al.* (6), using rabbit anti-rat Ig, found percentages of total sIg^+ cells in lymphoid tissues and sIg^+ $Thy1^+$ B cells in bone marrow that were comparable to the present results.

Another example of the immaturity of $Thy1^+$ B cells in the rat is our observation that only 22% of CRL in lymph node and 7% in spleen are $Thy1^+$. Expression of the receptor for the third component of complement (CR) occurs relatively late in B cell ontogeny in the rat, as in the mouse (31, 32), being present on fewer than 25% of sIg^+ cells in adult bone marrow and appearing in spleen and lymph node only after the first few weeks of life. Moreover, it has been reported that in the rat, the CR is present on all B cells that participate in the 19S antibody response (33) and the primary 7S response (34), as well as on a proportion of indirect PFC and cells that participate in the secondary 7S antibody response (33–35). In contrast, we have found that

TABLE IV

sIg^+ and $Thy1^+$ cells in TXBM rat tissues[a]

	Positive Cells			
	sIg[b]	Thy1[b]	sIg, Thy1/ Total sIg	sIg, Thy1/ Total Thy1
	%			
Bone marrow (normal)	7.8	35.4	95.0	20.8
Bone marrow (TXBM)	7.2	33.7	82.7	17.3
Spleen (normal)	36.8	13.4	22.8	62.4
Spleen (TXBM)	67.1	28.3	38.6	91.8
Lymph node (normal)	26.4	4.5	3.1	22.2
Lymph node (TXBM)	48.9	8.8	12.7	70.5

[a] Examined at 12 weeks of age.
[b] Expressed as a percentage of total nucleated cells.

TABLE V

Percentage of CRL which express sIg and Thy1 in adult rat spleen and lymph node[a]

	% Positive Cells		
	CRL[b]	sIg,[c] CRL/ Total CRL	Thy1[d], CRL/ Total CRL
Spleen	15.7 ± 1.9	98.5 ± 0.6	6.9 ± 2.2
Lymph node	14.0 ± 2.0	97.6 ± 1.0	22.2 ± 6.1

[a] Six to 8 weeks of age.
[b] Expressed as a percentage of total nucleated cells.
[c] sIg detected by TRITC $F(ab')_2$ rabbit anti-rat IgG.
[d] Thy1 detected by FITC $F(ab')_2$ rabbit anti-rat Thy1.

TABLE VI

Proportion of hemolytic PFC which bear Thy1 in rat spleen and lymph node[a]

Antigen	Direct PFC		Indirect PFC	
	Spleen	Lymph node	Spleen	Lymph node
SRBC	1/155	0/105	0/74	0/110
TNP-BSA	0/37	0/42	0/85	0/65

[a] Between three and eight rats were used for each determination. See *Materials and Methods* for immunization protocol.

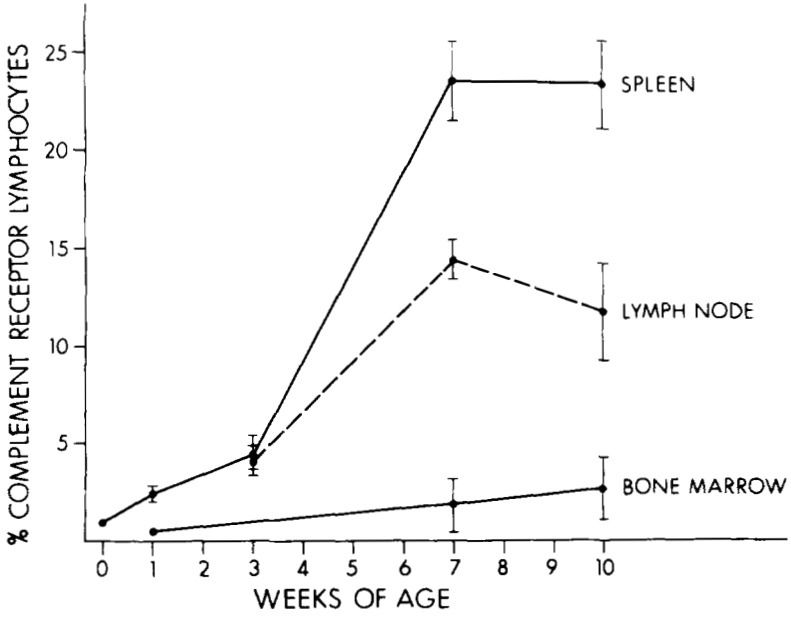

Figure 5. The ontogeny of CRL in rat bone marrow, spleen, and lymph node. Each point represents the percent of total nucleated cells (mean ± S.E.).

$Thy1^+$ B cells in the rat are not antibody-forming cells, as determined by direct or indirect PFC assays to SRBC or TNP antigens. This is true for both the CR^- and CR^+ subsets of $Thy1^+$ B cells.

It could be argued that sIg^+, $Thy1^+$ cells are not B cells, rather that they are T cells or other cell types that bear cytophilically or otherwise exogenously acquired immunoglobulin. This is not the case. Previous immunofluorescence studies have shown that T cells and sIg^+ cells in the rat are discrete, nonoverlapping populations (36); and that the vast majority of T cells in the rat are $Thy1^-$ (4, 7). Moreover, we and Hunt *et al.* (6) have shown that in rat bone marrow, approximately 75% of sIg^+ cells, which outnumber T cells 4:1 (36), are strongly $Thy1^+$, as are many bone marrow cells that form B lymphocyte colonies in semisolid medium *in vitro* (Goldschneider, unpublished observations).

Evidence that the sIg on $Thy1^+$ cells is not cytophilically acquired has been presented by Ritter *et al.* (7). In that study, sIg^+, $Thy1^+$ spleen cells from (Lewis × DA)F_1 hybrid rats were found to exhibit Ig light chain allelic exclusion, approximately half displaying the RI-1a allotype and half the RI-1b allotype. In addition, with the exception of experiments detailed in Table II, all of the antisera that were used in that and the present study were $F(ab')_2$ reagents, and all antisera were ultracentrifuged to remove aggregates, making it highly unlikely that cytophilic acquisition of these reagents occurred. The specificity of these reagents has been well documented (7, 16). It is also important to note that comparable percentages of sIg^+, $Thy1^+$ cells have been detected with rabbit heteroantiserum and mouse alloantiserum to Thy1.1 antigen.

Additional evidence that sIg^+, $Thy1^+$ cells are not T cells was provided by TXBM rats. This procedure, which selectively depletes T cells (36, 37), resulted in a 2-fold increase in the percentage of $Thy1^+$ cells in lymph node and spleen. As in irradiated, nonthymectomized rats, TXBM rats also had a significant increase in the percentage of sIg^+ cells that were $Thy1^+$. However, unlike the nonthymectomized rats, the ratio of $Thy1^+$:$Thy1^-$ B cells in TXBM rats did not return to normal with time. This suggests that $Thy1^+$ B cells may undergo maturational arrest in the absence of T cells, failing to differentiate efficiently into functionally mature, $Thy1^-$ B cells; or alternatively, that $Thy1^+$ and $Thy1^-$ B cells represent developmentally discrete cell lines that differ in their dependency on thymic humoral and/or T cell influence. Similar suggestions of B cell maturational defects have been reported in congenitally athymic (nu^+/nu^+) mice (38, 39).

The finding that sIg^+, $Thy1^+$ cells in rat lymph node and spleen are localized in the lymphoid follicles is important for two reasons. First, it confirms that sIg^+, $Thy1^+$ cells are B cells (36). Second, it suggests that the expression of Thy1 antigen on rat B cells is restricted to the antigen-independent and/or thymus-independent phases of differentiation. This inference is drawn from reports that the primary follicles of germfree (40, 41) or congenitally athymic mice (42) appear to be normally populated with lymphocytes. In contrast, germinal center cells, marginal zone cells, and plasma cells, all of which are $Thy1^-$ in the rat, are markedly reduced in such animals (43, 44), but can be restored by injection of antigen and/or T cells (45–47). Again, the results do not enable us to determine whether $Thy1^+$ and $Thy1^-$ B cells have a precursor-product relationship or whether they are members of two separate B cell lineages. Studies are in progress to test these developmental relationships and to define the functional capabilities of $Thy1^+$ B cells in the rat.

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