

A MONOCLONAL ANTIBODY THAT RECOGNIZES B CELLS AND B CELL PRECURSORS IN MICE*

BY ROBERT L. COFFMAN AND IRVING L. WEISSMAN

From the Laboratory of Experimental Oncology, Department of Pathology, Stanford University School of Medicine, Stanford, California 94305

B cells are produced from B cell precursors primarily in the bone marrow of adult mice (1), but relatively little is known about the differentiation steps that precede the appearance of cells with surface immunoglobulin (sIg)¹. A major difficulty with elucidating these steps has been the lack of well-defined surface markers which would facilitate the identification and isolation of B cell precursors. The alloantigen Lyb-2 (2) is expressed on B cells, a fraction of sIg⁻ bone marrow cells, and some putative pre-B cell tumors (3). Another B cell alloantigen, Lyb-1 (4), has a similar tissue distribution and, like Lyb-2, is expressed on some sIg⁻ bone marrow cells. Neither antigen, however, has been shown to be on B cell precursors.

Current techniques for producing monoclonal antibodies by cell fusion offer the prospect of defining new surface markers on B cell precursors and preparing large quantities of monospecific antibodies to those markers. Such antibodies would make possible, for the first time, the enumeration and purification of viable B cell precursors. The availability of highly enriched B cell precursors would permit a detailed exploration of early B cell differentiation at a cellular and molecular level. To raise such antibodies, we have initiated a program to make hybridomas with rats immunized with normal and neoplastic mouse B and pre-B cells. We report here a monoclonal antibody that recognizes a determinant on B cells and a fraction of sIg⁻ bone marrow cells that includes B cell precursors.

Materials and Methods

Hybridomas. A Lewis rat was immunized with four injections of 2×10^8 RAW 112 cells and the spleen was harvested 3 d after the last injection. The cells were fused in 38% polyethylene glycol with S194/5.XXO.BU-1 (bromodeoxyuridine-resistant) mouse myeloma cells and the mixture was plated immediately in hypoxanthine aminopterin thymidine (HAT) medium at 1.5×10^6 cells/1.5-ml well. When the hybrids reached a density of at least 2×10^5 cells/ml, the supernatant fluids were harvested and tested for immunofluorescent staining of bone marrow, thymus, and RAW 112 cells. RA3-2C2 was selected for further study because it stained RAW 112 and a subpopulation of bone marrow cells but not thymocytes. The cell line was subcloned by limiting dilutions before further use.

Animals. BALB/c mice and Lewis rats were bred in our own colony. Mice of either sex were

* Supported by grants AI 09072 from the National Institutes of Health, CP 91011 from the National Cancer Institute, and IM56 from the American Cancer Society. R. L. C. was supported by fellowships CA 09302 and CA 06429 from the National Institutes of Health.

¹ *Abbreviations used in this paper:* BSS, modified Hanks' basic salts solution; CFU-s, spleen colony-forming unit; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; KLH, keyhole limpet hemocyanin; sIg, surface immunoglobulin; TRITC, tetramethylrhodamine isothiocyanate.

used in these experiments at 6–10 wk of age, except the recipient mice for the spleen colony-forming unit (CFU-s) assay, which were 4–6 mo of age.

Antisera. Rabbit anti- μ chain and anti- κ antisera were specifically eluted from myeloma protein immunoadsorbent columns and have been characterized previously (5). Specifically eluted rabbit anti-keyhole limpet hemocyanin (KLH) was used as a control first stage. Goat anti-rabbit Ig was specifically eluted from a rabbit Ig column and conjugated with fluorescein isothiocyanate (FITC) to yield a ratio of four fluorescein molecules per molecule of IgG. Rabbit anti-rat Ig was passed first through a mouse Ig column to remove cross-reactive antibodies, and then eluted from a rat Ig column. It was conjugated with FITC to yield a ratio of five fluorescein molecules per molecule of IgG. RA3-2C2 was partially purified from tissue culture supernate by precipitation twice with 40% saturated ammonium sulfate, followed by gel filtration on Sepharose 6B. This material was either directly conjugated with fluorescein or was used with fluorescent rabbit anti-rat Ig. This preparation was ~25–50% antibody and was conjugated with FITC to a ratio of 30 fluorescein molecules per molecule of IgM. A rat monoclonal IgM with no apparent cell-binding activity was purified and fluoresceinated in the same way and was used as a negative control. Rat anti-mouse Thy-1.2 was obtained from the supernate of the hybridoma cell line 30-H12 (6).

Immunofluorescent Staining. All staining and cell sorting was done in Hanks' basic salts solution (BSS) with 10^{-3} M sodium azide and 5% fetal calf or calf serum and without phenol red. Bone marrow cell suspensions were prepared by flushing femurs with BSS and pipetting the suspension through a fine stainless steel screen. Spleen, lymph node, and thymus suspensions were prepared by mincing the organ and pressing it through a fine screen. All cell suspensions were treated with a solution of 0.155 M NH_4Cl , 0.01 M KHCO_3 , and 10^{-4} M EDTA to lyse erythrocytes. Cell pellets were resuspended at 10^8 cells/ml in the appropriate concentration of first or second stage antibody and incubated on ice for 15–30 min. The cell suspension was then diluted 20-fold in BSS and centrifuged through a layer of calf serum.

Flow Cytometry Analysis and Cell Separations. All flow cytometry and cell sorting was done on a fluorescence-activated cell sorter (FACS III, Becton-Dickinson Electronic Laboratories, Mountain View, Calif.). Viable and nonviable cells were discriminated on the basis of light scatter (7) and only the fluorescence of viable cells was considered. Cell sorting was performed at a rate of 2,000–3,000 cells/s. Positive and negative gates were separated by 10–15 channels in order to improve the purity of the fractions. Negative fractions contained 1–3% positive cells and positive fractions contained 2–8% negative cells. Cells were maintained at 0°C in 10^{-3} M sodium azide throughout the sorting procedure. "Panning" on antibody-coated petri dishes was done by the procedure of Wysocki and Sato (8). For the removal of B cells, 100-cm dishes were coated with 20 μg each of rabbit anti- μ and anti- κ . Purified RA3-2C2 was used at 50 μg /dish for panning.

In Vitro Cultures. Cells were cultured for 2 d at a density of 5×10^6 cells/ml in Iscove's medium (gift of Dr. J. D. Watson, University of California, Irvine) plus 10% fetal calf serum. Cells were plated in 0.2-ml tissue culture wells and grown in 8% CO_2 . Dead cells in these cultures exhibited significant autofluorescence and nonspecific fluorescent staining and could not be satisfactorily gated out on the FACS. As a consequence, stained cultures were scored in a fluorescence microscope where live and dead cells could be resolved on the basis of morphology and staining pattern. At least 600 cells were counted for each sample. Tetramethylrhodamine isothiocyanate (TRITC) labeling of cells was done as described previously by Butcher and Weissman (9).

Spleen Colony-forming Unit Assay. This assay was performed essentially as described by Till and McCulloch (10). BALB/c mice were given 850 rad of x-ray irradiation before intravenous injection of bone marrow cells.

Plaque-forming Cell Assay. Plaque-forming cells to sheep erythrocytes were enumerated by the method of Mishell and Dutton (11). IgG plaques were developed by adding rabbit anti-mouse Ig to the complement solution. Mice were immunized intraperitoneally with 0.2 ml of 10% sheep erythrocytes 5 d before use for the primary response, and 60 d and again 5 d before use for the secondary response.

Results

Production of RA3-2C2 Monoclonal Antibody. The monoclonal antibody RA3-2C2 was selected from the products of a fusion of mouse myeloma cells with spleen cells from a rat immunized with the cell line RAW 112. RAW 112 is an Abelson murine leukemia virus-induced tumor with a pattern of responsiveness to B cell mitogens which suggests it is a pre-B cell tumor (12). Tumor cells were used as the immunogen in preference to normal tissues in order to provide a relatively enriched source of pre-B cell antigens.

Fused cells were plated with HAT selection medium in 1.5-ml tissue culture wells and grown until the cells reached at least 2×10^5 cells per well. The supernatant fluids were then harvested and screened by immunofluorescent staining of RAW 112 cells. All positive supernatant fluids were further examined by immunofluorescent staining and FACS analysis on normal bone marrow and thymus cell suspensions. RA3-2C2 was selected for further study because it stained only 30% of bone marrow cells and did not stain thymocytes.

The RA3-2C2 antibody was shown serologically to be an IgM and by gel filtration to be a polymeric (probably 19s) Ig molecule. No detectable amount of mouse κ -chain was incorporated into the active molecule. The antibody bound well to *Staphylococcus aureus* bacteria and purified protein A at pH 7.2, and these could be used for, respectively, immunoprecipitation with and purification of RA3-2C2 (R. Coffman, unpublished results).

Immunofluorescent Staining of Lymphoid Tissue with RA3-2C2. The percentages of cells that stain with RA3-2C2 in lymphoid tissues is shown in Table I. The percentages of cells positive with anti- κ (B cells) or anti-Thy 1 (T cells) are shown for comparison. The monoclonal antibody was used either as a direct fluorescein conjugate or with a fluorescent rabbit anti-rat Ig second stage. A rat IgM monoclonal antibody with no detectable cell binding activity was used as a control for both one-stage and two-stage immunofluorescent stainings. Controls usually contained 1–3% of cells in the positive channels. RA3-2C2 stains approximately the same number of cells as anti- κ in spleen and lymph nodes, but stains ~20% more than anti- κ in the bone marrow. No staining was detected in the thymus. The median fluorescence intensity of RA3-2C2 on spleen and lymph node cells is about twice that on bone marrow cells.

TABLE I
Immunofluorescent Staining of Lymphoid Organs with RA3-2C2

Cell suspension from	Cells stained with*		
	RA3-2C2‡	Anti- κ	Anti-Thy-1
	%	%	%
Bone marrow	32	10	2
Spleen	45	52	28
Lymph node	18	19	67
Thymus	0	0	97

* Percent-positive cells minus percent-positive in negative controls (see Materials and Methods). Negative controls ranged from 1 to 3% positive.

‡ Spleen and lymph nodes were stained with fluorescein-conjugated RA3-2C2. All other stains were done with fluorescein-conjugated rabbit anti-rat Ig or goat anti-rabbit Ig.

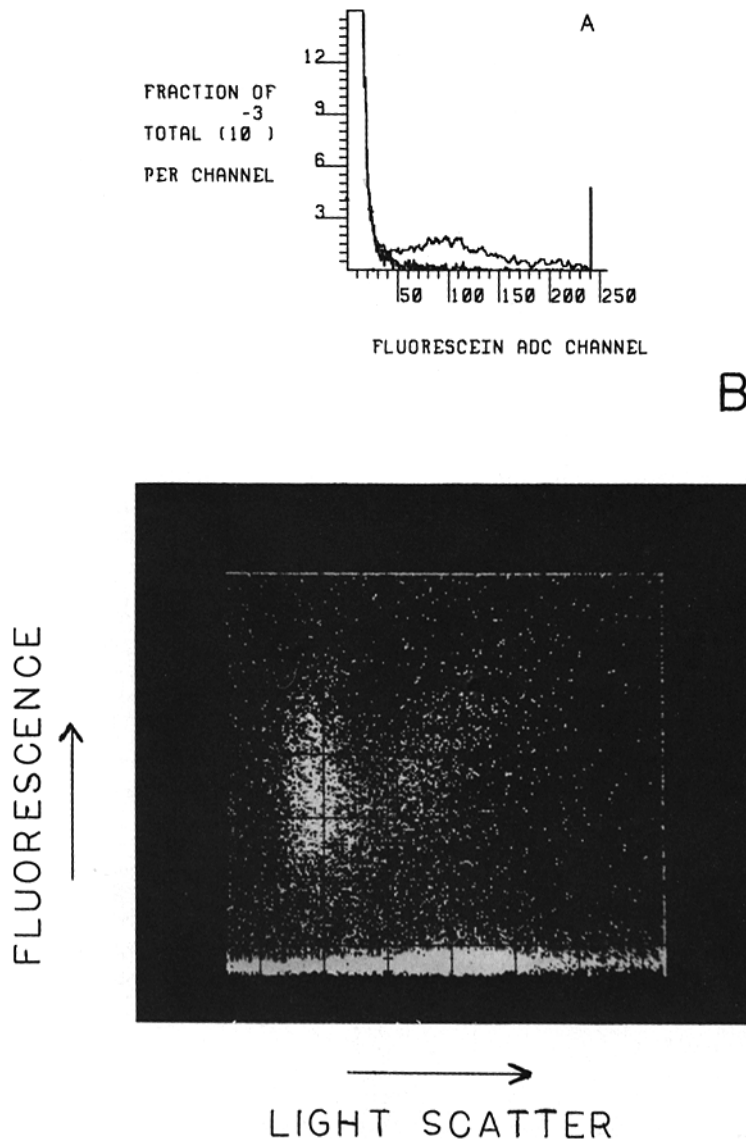


FIG. 1. Immunofluorescent staining of bone marrow cells with RA3-2C2. (A) Bone marrow cells stained with RA3-2C2 plus fluorescein-conjugated rabbit anti-rat Ig (upper curve) compared with cells stained with a control rat IgM plus the same second stage. (B) Two-parameter display of fluorescence intensity vs. light scatter (which is approximately proportional to cell size). Each dot represents the data for a single cell.

The staining profile of RA3-2C2 on bone marrow cells is shown in Fig. 1A, with the control staining shown for comparison. There is a clear bimodal distribution which permits reasonably accurate enumeration and separation of positive and negative fractions. A two-parameter "dot-plot" of fluorescence vs. light scatter (which is approximately proportional to size in bone marrow cells) shows that both large and small cell fractions include RA3-2C2⁺ cells (Fig. 1B). 65–75% of the RA3-2C2⁺ bone

marrow cells are in the small cell fraction, with the remainder being in the medium to large cell fraction. The morphology of RA3-2C2⁺ bone marrow cells was analyzed by staining cytocentrifuge smears of FACS-purified cells with Wright's stain. The RA3-2C2⁺ fraction was 68% small lymphocytes, and 25% medium to large blast cells. The remaining 7% included a variety of other cell types and probably represented RA3-2C2⁻ contaminants because the RA3-2C2⁺ fraction contained 8% negative cells. The RA3-2C2⁻ fraction was significantly depleted of lymphocytes; thus, RA3-2C2 detects an antigen on most bone marrow lymphocytes.

RA3-2C2 Is on All B Cells and a Fraction of sIg⁻ Bone Marrow Cells. The preceding results suggested that RA3-2C2 recognized primarily B cells and, in the bone marrow, might also include B cell precursors. To test this, sIg⁻ and RA3-2C2⁻ fractions of spleen and bone marrow were isolated by cell sorting, then restained with anti- κ or RA3-2C2 (Table II). Removal of the sIg⁺ cells from the spleen removes virtually all RA3-2C2⁺ cells, and at the same time removal of the RA3-2C2⁺ cells removes all sIg⁺ cells. Therefore, in the spleen, all B cells are RA3-2C2⁺ and all RA3-2C2⁺ cells are B cells. In the bone marrow, removal of RA3-2C2⁺ cells also removes all B cells, but removal of sIg⁺ cells leaves a population that is still 19% RA3-2C2⁺. In summary, ~10% of bone marrow cells are B cells and nearly all are small lymphocytes. Another 10–12% of bone marrow cells are small sIg⁻, RA3-2C2⁺ lymphocytes, and 8–10% are medium to large sIg⁻, RA3-2C2⁺ cells.

RA3-2C2⁺, sIg⁻ Bone Marrow Cells Include B Cell Precursors. To demonstrate that the RA3-2C2⁺, sIg⁻ bone marrow fraction included B cell precursors, we used a short-term in vitro culture system in which B cells arise from sIg⁻ precursors. Briefly, bone marrow cells are depleted of sIg⁺ cells by panning on petri dishes coated with anti- μ and anti- κ antibodies (8). The nonadhering sIg⁻ cells were cultured in Iscove's medium plus 10% fetal calf serum for 2 d, then stained with a mixture of rabbit anti- μ and anti- κ to detect newly formed B cells. The mixture of anti- κ and anti- μ was used to increase the staining intensity and make visual scoring of positive cells more sensitive and accurate. In some experiments, aliquots were also stained with anti- κ or

TABLE II
Immunofluorescent Staining of sIg⁻ and RA3-2C2⁻ Spleen and Bone Marrow Cells

Cell fraction	Cells stained with*	
	Anti- κ	RA3-2C2‡
	%	%
Unseparated spleen cells	44	40
sIg ⁻ spleen cells	2	4
RA3-2C2 ⁻ spleen cells	1	3
Unseparated bone marrow cells	10	27
sIg ⁻ bone marrow cells	0	19
RA3-2C2 ⁻ bone marrow cells	0	3

* Percent-positive cells minus percent-positive in negative controls in sorted fractions, the negative control includes 1–2% positive contaminants in addition to nonspecific staining of negative cells, which was 1–3% positive.

‡ Spleen cell fractions were stained with fluorescein-conjugated RA3-2C2, bone marrow fractions with a fluorescein-conjugated rabbit anti-rat Ig.

anti- μ alone. In all cases, the percent of positive cells was within 10% of the percent positive with the mixture of antisera, demonstrating that most sIg⁺ cells expressed both μ - and κ -chains.

When the RA3-2C2⁺ cells are removed from sIg⁻ bone marrow by cell sorting, this B cell precursor activity is removed (Table III). Stained but unsorted cultures give rise to normal numbers of B cells, so the staining procedure is not responsible for the loss of precursor activity. When RA3-2C2⁺ cells are removed from Ig⁻ bone marrow by a subsequent panning on purified RA3-2C2-coated petri dishes, B cell precursor activity is eliminated from the unbound fraction. This procedure causes less cell death and suggests that the process of sorting does not preferentially kill B cell precursors. Attempts to culture the purified RA3-2C2⁺, sIg⁻ fraction have been less successful because, when purified, these cells usually die within 2 d in vitro. This suggests that either an RA3-2C2⁻ accessory cell is necessary for the survival, and possibly the differentiation, of the RA3-2C2⁺ cells, or that the B cell precursors are RA3-2C2⁻ but require an RA3-2C2⁺ accessory cell for their differentiation to proceed. To test these possibilities, sIg⁻ and RA3-2C2⁻, sIg⁻ bone marrow cells were marked by labeling with TRITC (9) and the labeled cells were then cultured with a threefold excess of unlabeled sIg⁻ bone marrow cells. After 2 d, the cultured cells were stained with a mixture of anti- μ and anti- κ , followed by a fluorescein-conjugated second stage, and the positive cells were counted both for the unlabeled and the TRITC-labeled cells. For comparison, portions of the same fractions were cultured without labeling and mixing. RA3-2C2⁻, sIg⁻ cells do not differentiate into B cells even in the presence of unlabeled RA3-2C2⁺ cells (Table IV). That these cultures do provide the proper environment for this differentiation is shown by the fact that the unlabeled sIg⁻ bone marrow cells in the same cultures give rise to the expected percentage of sIg⁺ cells. This experiment demonstrates that removal of the RA3-2C2⁺ cells from the sIg⁻ bone marrow removes the B cell precursors themselves and not just accessory cells. TRITC conjugation does not interfere with this B cell differentiation because cultures of labeled sIg⁻ cells give rise to the expected number of B cells either in mixed culture (Table IV) or when cultured alone (data not shown).

RA3-2C2 Does Not Recognize the Pluripotent Hematopoietic Stem Cell. The RA3-2C2 determinant would be especially useful if it were expressed only on cells committed to the B cell lineage and not on the pluripotent stem cell from which B cells, as well as

TABLE III
Bone Marrow Cells that Express sIg in Vitro Are RA3-2C2⁺ and sIg⁻

Cell fraction	Cells stained with*	
	Anti- μ + Anti- κ	Anti-KLH‡
	%	%
Ig ⁻ bone marrow	10.3	1.4
Ig ⁻ bone marrow, stained with 2C2	10.5	2.1
Ig ⁻ , 2C2 ⁻ bone marrow, sorted	0	0
Ig ⁻ , 2C2 ⁻ bone marrow, panned	1.4	1.2

* Cells were stained and counted after 2 d in vitro. At least 600 viable cells were counted in each sample. The data are pooled from three separate experiments.

‡ Control for nonspecific staining.

TABLE IV
B Cell Precursors, Rather than Accessory Cells, Are Absent in RA3-2C2⁻ Bone Marrow Cells

Cell fraction	Unlabeled cell fraction cultured alone	Rhodamine-labeled cell fraction* + unlabeled Ig ⁻ bone marrow cells	
	Ig ⁺ -BKG‡	Labeled cells Ig ⁺ -BKG	Unlabeled cells Ig ⁺ -BKG
	%	%	%
Ig ⁻ bone marrow	8.4	11.0	7.6
Ig ⁻ RA3-2C2 ⁻ bone marrow, sorted	0	0	10.8
Ig ⁻ RA3-2C2 ⁻ bone marrow, panned	0.8	1.5	9.1

* The cell fraction was split with one portion being cultured without labeling or mixing, and the other portion being labeled with TRITC and mixed on a 1:3 ratio with unlabeled Ig⁻ bone marrow cells. All cultures were maintained at 5×10^6 total cells/ml.

‡ Percent of cells stained with a mixture of anti- μ and anti- κ minus the percent stained with anti-KLH.

TABLE V
CFU-s Cells Are RA3-2C2⁻

Mice restored with	CFU-s*	
	Experiment 1	Experiment 2
10^5 bone marrow cells	53 ± 2	47 ± 6
10^5 bone marrow cells, stained with RA3-2C2 and rabbit anti-rat Ig	39 ± 7	41 ± 10
3×10^4 RA3-2C2 positive bone marrow cells	5 ± 1	1 ± 1
6×10^4 RA3-2C2 negative bone marrow cells	45 ± 5	43 ± 9

* Each group comprised four–six recipient spleens.

TABLE VI
RA3-2C2 Stains Most Plaque-forming Cells

Cells	Plaque-forming cells/ 10^6 cells	
	Primary response IgM plaques*	Secondary response IgG plaques‡
Unsorted spleen	900	450
RA3-2C2 ⁺ spleen	1,170	980
RA3-2C2 ⁻ spleen	430	160

* Animals were immunized with sheep erythrocytes 5 d before sacrifice.

‡ Animals were immunized with sheep erythrocytes 60 and 5 d before sacrifice.

other hemolymphoid cells, derive (13). This was tested by sorting bone marrow into RA3-2C2 positive and negative fractions and assaying each for the presence of CFU-s (10). Sorting gates were set so that 10 channels at the boundary between positive and negative staining were not included in either fraction. This was done to obtain suitable purity of the sorted fractions and resulted in the loss of 5–7% of the

cell suspension. Lethally irradiated mice were injected with 10^5 bone marrow cells or the RA3-2C2 positive or negative fractions from 10^5 bone marrow cells, and the spleen colonies were counted 9 d later. As shown in Table V, virtually all CFU-s are found in the RA3-2C2⁻ fraction. Stained but not sorted bone marrow cells do not have significantly reduced numbers of CFU-s; their absence in the positive fraction is not due to inhibition by the antibody.

RA3-2C2 Recognizes Most Plasma Cells. The recognition by RA3-2C2 of the terminal stage of the B cell lineage, the plasma cell was tested by sorting RA3-2C2 positive and negative fractions from sheep erythrocyte-primed spleen cells. The number of sheep erythrocyte-specific antibody-forming cells in each fraction was determined by the Jerne plaque-forming cell assay. The results of one of three such experiments are shown in Table VI. RA3-2C2 appears to recognize the majority of primary IgM and secondary IgG plaque-forming cells, but there are significant numbers of each in the RA3-2C2⁻ fraction. The presence of plaque-forming cells in the RA3-2C2⁻ fraction is not due to contamination by RA3-2C2⁺ cells, since both negative fractions contained only 1% RA3-2C2⁺. This may indicate a significant heterogeneity among plasma cells, or it may mean that plasma cells generally bind less RA3-2C2 than B cells, and some of them bind too little to be resolved from RA3-2C2⁻ cells.

Discussion

The determinant recognized by the monoclonal antibody, RA3-2C2, is expressed on a spectrum of cell types within the B cell lineage. At the same time, its expression appears to be restricted largely, if not entirely, to the B lineage. It is expressed on more cell types than other commonly used B cell markers such as sIg and Ia, and may prove to be a more useful marker for members of this lineage. Some care is necessary, however, to get accurate staining percentages, especially when the analysis is done by flow cytometry. Perhaps because the antibody is a multivalent IgM, it causes significant agglutination of some positive cells when used with an anti-rat Ig second stage. Flow cytometers, such as the FACS, filter out these aggregates so they cannot clog the nozzle orifice, and the remaining single-cell suspension is therefore relatively depleted of positive cells. For example, until this artifact was noticed, spleen cell suspensions appeared to be only 20% RA3-2C2⁺ on the FACS. The problem can be minimized by using directly fluoresceinated monoclonal antibody and resuspending the cells immediately before analysis. The phenomenon is observed primarily with spleen and lymph node cells, and little, if any, agglutination is observed with bone marrow cells.

Approximately 10% of nucleated bone marrow cells are RA3-2C2⁺, sIg⁺ lymphocytes. Another 20% of bone marrow cells are RA3-2C2⁺, sIg⁻, and are predominantly lymphoid in morphology. The total lymphoid compartment of adult bone marrow has been estimated by others to consist of about 25% of nucleated cells (14), with approximately equal numbers of sIg⁺ and sIg⁻ small lymphocytes (15, 16). Furthermore, the number of B cell precursors, as measured by the presence of cytoplasmic chains, is approximately equal to the number of sIg⁺ B cells (17). These estimates, from a number of laboratories, are consistent with the estimate of the lymphoid compartment as judged by the RA3-2C2⁺ cells. At least two types of cells are present in the sIg⁻, RA3-2C2⁺ bone marrow fraction. The majority are small lymphocytes which comprise 50–60% of the sIg⁻, RA3-2C2⁺ fraction, while the remainder are

larger cells with morphologically undifferentiated nuclei and relatively little cytoplasm. These larger cells most likely include the transitional lymphoid cells described by Miller and Osmond (14), but they may include immature stages in other hematopoietic lineages as well. Both small and large sIg⁻ lymphoid populations have been shown to include cells containing cytoplasmic μ -chains (17). Although the labeling kinetics of these two cell types were consistent with the idea that the larger cell is a precursor for the smaller one, a direct precursor-product relationship between the two cell types has not yet been demonstrated.

The *in vitro* culture system for the generation of B cells from B cell precursors is very similar to ones described previously (15, 18, 19). It seems likely that only relatively mature precursors give rise to sIg⁺ cells under these conditions. Most of the B cells are generated within the first 24 h and most of the activity resides in the small cell fraction of bone marrow (R. Coffman, unpublished results). B cell precursor activity has been demonstrated in large bone marrow cells as well, but the differentiation of these cells into antigen- or mitogen-responsive B cells requires 4–5 d *in vitro* (20) and possibly longer *in vivo* (21).

Direct fluorochrome conjugation of cells has been used in our laboratory to study lymphocyte homing and thymus cell maturation. These studies show that FITC or TRITC conjugation of lymphoid cells does not impair either specific lymphocyte migration (22) or graft vs. host reactivity (9). Similarly, TRITC conjugation does not impair the ability of B cell precursors to express sIg. After 2 d, some loss of TRITC occurs, but all cells that were initially labeled can still be readily detected.

The RA3-2C2 determinant is not expressed on the pluripotent hematopoietic stem cell, suggesting that it is expressed later in the differentiation pathway, perhaps at about the same time as the commitment to the B cell lineage. This assumes that B cells derive from the same cell which gives rise to the spleen colonies. This assumption is supported by studies in which unique, radiation-induced, chromosome markers are found both in spleen colonies and in T and B cell blasts (13). Although B cells are rare in spleen colonies, Lala and Johnson have shown that they are part of the same clone as the rest of the colony (23).

The nature of the surface molecule bearing the RA3-2C2 determinant is unclear at present. RA3-2C2 precipitates a 50,000-mol wt molecule (P50) from biosynthetically labeled bone marrow, spleen, thymus cells, and from Abelson murine leukemia virus-transformed cell lines (24). The synthesis of this protein is greatly increased in some transformed cells and is expressed by many nonlymphoid tumors as well. It is not known, however, whether the P50 molecule is expressed on the cell surface, because no P50 is detectable in immunoprecipitates of lactoperoxidase-catalyzed surface-radioiodinated cells.

The reason for producing monoclonal antibodies directed against pre-B tumors was to isolate antibodies to determinants on normal B cell precursors. The ideal antibody would perhaps be one that was specific for these precursors and did not recognize sIg⁺ B cells, but no such antibody has been found in three fusion experiments. For the purposes of enumerating and isolating pre-B cells, however, the B lineage specific antibody described here may be just as useful. More than 90% of B cells can be routinely removed from bone marrow by the rapid and gentle technique of panning on anti- μ - and anti- κ -coated petri dishes. When necessary, even better depletions of B cells can be achieved with the FACS. After depletion of sIg⁺ cells from the bone

marrow, the remaining RA3-2C2⁺ cells appear to be largely, if not entirely, B cell precursors. These cells can then be isolated with 90–95% purity on the FACS. Experiments to examine Ig biosynthesis and Ig gene organization in these cells are currently in progress.

Summary

The monoclonal antibody, RA3-2C2, appears to be specific for cells within the B cell lineage. This antibody does not recognize thymocytes, peripheral T cells, or nonlymphoid hematopoietic cells in the spleen or bone marrow. Nor does it recognize the pluripotent hematopoietic stem cells, the spleen colony-forming unit. All sIg⁺ B cells and most plasma cells are RA3-2C2⁺. In addition, ~20% of nucleated bone marrow cells are RA3-2C2⁺ but sIg⁻. This population contains B cell precursors that can give rise to sIg⁺ cells within 2 d in vitro.

We wish to thank Francis Assisi and Trygve Statte for their excellent assistance on the FACS.

Received for publication 6 October 1980.

References

1. Osmond, D. G. 1975. Formation and maturation of bone marrow lymphocytes. *J. Reticuloendothel. Soc.* **17**:99.
2. Sato, H., and E. A. Boyse. 1976. A new alloantigen expressed selectively on B cells: the LyB-2 system. *Immunogenetics.* **3**:565.
3. Silverstone, A. E., N. Rosenberg, V. L. Sato, M. P. Scheid, E. A. Boyse, and D. Baltimore. 1978. Correlating terminal deoxynucleotidyl transferase and cell-surface markers in the pathway of lymphocyte ontogeny. In *Differentiation of Normal and Neoplastic Hematopoietic Cells*. B. Clarkson, J. E. Till, and P. Marks, editors. Cold Spring Harbor Lab., Cold Spring Harbor, New York. 433.
4. McKenzie, I. F. C., and G. D. Snell. 1975. Ly-4.2: a cell membrane alloantigen of murine B lymphocytes. I. Population studies. *J. Immunol.* **114**:848.
5. Coffman, R. L., and M. Cohn. 1977. The class of surface immunoglobulin on virgin and memory B lymphocytes. *J. Immunol.* **118**:1806.
6. Ledbetter, J. A., and L. A. Herzenberg. 1979. Xenogenic monoclonal antibodies to mouse differentiation antigens. *Immunol. Rev.* **47**:63.
7. Loken, M. R., and L. A. Herzenberg. 1975. Analysis of cell populations with a fluorescence actuated cell sorter. *Ann. N. Y. Acad. Sci.* **254**:163.
8. Wysocki, L. J., and V. L. Sato. 1978. "Panning" for lymphocytes: a method for cell selection. *Proc. Natl. Acad. Sci. U. S. A.* **75**:2844.
9. Butcher, E. C., and I. L. Weissman. 1980. Direct fluorescent labeling of cells with fluorescein or rhodamine isothiocyanate. I. Technical aspects. *J. Immunol. Methods.* **37**:97.
10. Till, J. E., and E. A. McCulloch. 1961. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* **14**:213.
11. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126**:423.
12. Ralph, P., I. Nakoinz, and W. C. Raschke. 1974. Lymphosarcoma cell growth is selectively inhibited by B lymphocyte mitogens: LPS, dextran sulfate and PPD. *Biochem. Biophys. Res. Commun.* **61**:1268.
13. Abramson, S., R. G. Miller, and R. A. Phillips. 1977. The identification in adult bone

- marrow or pluripotent and restricted stem cells of the myeloid and lymphoid systems. *J. Exp. Med.* **145**:1567.
14. Miller, S. C., and D. G. Osmond. 1974. Quantitative changes with age in bone marrow cell populations of C3H mice. *Exp. Hematol.* **2**:227.
 15. Ryser, J-E., and P. Vassali. 1974. Mouse bone marrow lymphocytes and their differentiation. *J. Immunol.* **113**:719.
 16. Osmond, D. G., and G. J. V. Nossal. 1974. Differentiation of lymphocytes in mouse bone marrow. II. Kinetics of maturation and renewal of antiglobulin-binding cells studied by double labeling. *Cell. Immunol.* **13**:132.
 17. Owen, J. J. T., P. E. Wright, S. Habu, M. C. Raff, and M. D. Cooper. 1977. Studies on the generation of B lymphocytes in fetal liver and bone marrow. *J. Immunol.* **118**:2067.
 18. Fairchild, S. S., and J. J. Cohen. 1978. B lymphocyte precursors. I. Induction of lipopolysaccharide responsiveness and surface immunoglobulin expression *in vitro*. *J. Immunol.* **121**:1227.
 19. Pike, B. L., and G. J. V. Nossal. 1979. Mechanisms of clonal abortion tolerogenesis. III. Antigen abrogates functional maturation of surface immunoglobulin-negative adult bone marrow lymphocytes. *Eur. J. Immunol.* **9**:708.
 20. Lau, C. Y., F. Melchers, R. G. Miller, and R. A. Phillips. 1979. *In vitro* differentiation of B lymphocytes from pre-B cells. *J. Immunol.* **122**:1273.
 21. Lafleur, L., R. G. Miller, and R. A. Phillips. 1972. A quantitative assay for the progenitors of bone marrow-associated lymphocytes. *J. Exp. Med.* **135**:1363.
 22. Butcher, E. C., R. C. Scollay, and I. L. Weissman. 1980. Direct fluorescent labeling of cells with fluorescein or rhodamine isothiocyanate. II. Potential application to studies of lymphocyte migration and maturation. *J. Immunol. Methods.* **37**:109.
 23. Lala, P. K., and G. R. Johnson. 1978. Monoclonal origin of B lymphocyte colony-forming cells in spleen colony-forming by multipotential hematopoietic spleen cells. *J. Exp. Med.* **148**:1468.
 24. Rotter, V., O. N. Witte, R. L. Coffman, and D. Baltimore. 1980. Abelson murine leukemia virus-induced tumors elicit antibodies against a host cell protein, P50. *J. Virol.* **36**:547.