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Differentiation Antigen on Murine B Lymphocytes Defined by Monoclonal Antibodies

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SUMMARY **Spleen cells from an AKR/J × DBA/2J F_1 mouse immunized with NZB/BIN spleen cells were fused with SP2/0-Ag14. Two hybrid cell lines, B220-1 and B220-2, were established that secreted antibody to the B-lineage specific B220 antigen. B220-1 and B220-2 are present on 45-55% of splenic and bone marrow lymphocytes and absent from thymus. By flow cytometry, all immunoglobulin-bearing cells were stained by these monoclonal antibodies. Although these monoclonals do not stain thymocytes, they do react weakly with Lyt-2^+ peripheral T cells. Dual parameter analysis of B lymphocytes using RA_3-3A1 or 14.8 show that these monoclonals recognized the same population. Prior incubation with RA_3-3A1 or 14.8 was unable to completely block the binding of B220-1 or B220-2, implying that the epitopes recognized are different from the previously described monoclonal antibodies. Immunoprecipitation of the splenic lymphocyte reveals a molecule which migrates on SDS-PAGE as a single band with MW of 220,000 daltons. Expression of the distinct antigens recognized by B220-1 and B220-2 varied among mouse strains, indicating previously unappreciated polymorphism of the B220 molecule. These monoclonals are useful for cytotoxic elimination of B cells and for three-color flow cytometry.**

Lymphocytes are divided into two major populations, B and T, according to their differentiation pathways and functional potential. Monoclonal antibodies which recognize lymphocyte differentiation antigens[1-3] have proved very useful in the delineation of differentiation pathways[4] and analysis of lymphocyte subsets.[5] In an attempt to obtain monoclonal antibodies specific for lymphoid differentiation antigens of autoimmune New Zealand Black (NZB) mice, we produced two clones that recognize the B-lineage specific B220 antigen, one of which is autoreactive since it reacts strongly with both parent mouse strains.

MATERIALS AND METHODS

Animals

AKR/J × DBA/2J F_1 and strains with the suffix /J were obtained from Jackson Laboratory, Bar Harbor, ME. NZB/BlN and all other strains came from the Small Animal Section, Division of Research Services, NIH, Bethesda, MD.

Production and screening of hybridomas

AKR/J × DBA/2J F_1 mice, which share the H-2^d haplotype with NZB, were immunized intravenously and boosted 3 weeks later with 10^7 NZB spleen cells. Three days after boosting their serum was tested for antibody to NZB spleen cells. Spleen cells from a mouse with a high antibody titer (staining shown in Fig. 1) were fused with an equal number of SP2/0-Ag14 cells[6] in the presence of 30% polyethylene glycol (MW 1000, Baker Chemical Corp., Phillipburg, NJ) as described by Oi and Herzenberg.[7] The fusion mixture was resuspended in selective HAT medium containing DME with 10% NCTC 109 (Microbiological Associates, Bethesda, MD), 1% non-essential amino acids (GIBCO), 20% fetal calf serum, 0.2 units/ml insulin, 1 mM oxaloacetic acid, 0.5 mM sodium pyruvate, 0.03% gluta-

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mine, 100 μM hypoxanthine, 1 μM aminopterin, and 30 μM thymidine (all from Sigma, St. Louis, MO) and distributed into 96 well microtiter plates at 2×10^5 spleen cells in 100 μl per well. Three days later 100 μl of fresh medium was added. Three weeks later 100 μl of supernatant from wells showing hybrid cell growth were incubated with 10^6 NZB spleen cells. After washing, the cells were exposed to fluoresceinated, affinity-purified goat anti-mouse IgG and analyzed by flow cytometry. Hybrid cells from the positive wells were cloned by limiting dilution with 2000 R irradiated BALB/c thymocytes. Selected clones were recloned using endothelial growth supplement[8] at 200 μg/ml (Collaborative Research, Lexington, MA).

Antibody purification and conjugation

The monoclonal antibodies were purified from the culture supernatant by protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) affinity chromatography, eluting with glycine-HCl, pH 3.0. The purified protein was conjugated with fluorescein isothiocyanate (Research Organics, Cleveland, OH) and biotin succinimide (Sigma Chemical, St. Louis, MO). Avidin (Sigma) was coupled to sulforhodamine-101 phosphoryl chloride (" Texas Red ", Molecular Probes, Junction City, OR). All reagents were centrifuged at 130,000 g for 20 minutes in a Beckman airfuge to remove aggregates. The immunoglobulin isotype was determined with class-specific antisera.

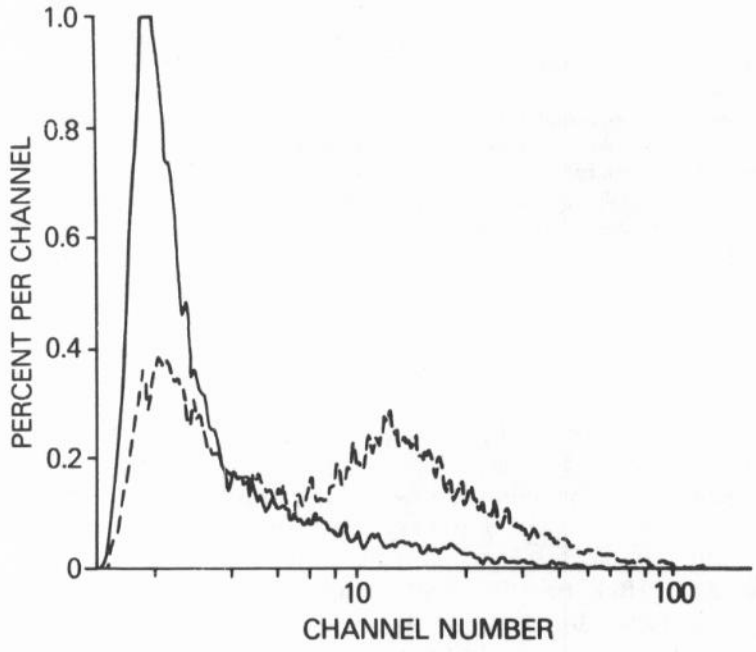

Fig. 1 Immunofluorescent staining of NZB spleen cells with serum from an AKR/J × DBA/2N F_1 mouse injected with NZB spleen cells (dashed line) compared to serum from a control, saline-injected animal (solid line). Staining was developed by fluoresceinated goat anti-mouse Ig preabsorbed with mouse spleen cells.

Monoclonal antibodies to known cell surface antigens

Hybridoma cell lines RA_3-3A1/6.1 (rat IgM anti-B220),[3] 14.8 (rat IgG_{2a} anti-B220)[9] and 331.12 (rat IgG_{2a} anti-mouse IgM)[10] were obtained from the American Type Culture Collection, Bethesda, MD. Hybridoma cell lines secreting monoclonal anti-IgD (mouse IgG_{2a}, clone 10.4-22), anti-Lyt2 (rat IgG_2, clone 53-6.7),[2] and anti-L3T4 (rat IgG_{2a}, clone GK1.5)[11] were kindly provided by Drs. L. Herzenberg, J. Ledbetter, and F. Fitch, respectively. These monoclonal antibodies were harvested from culture supernatants by ammonium sulfate precipitation followed by DEAE ion exchange and gel filtration chromatography. Monoclonal mouse anti-rat κ (clone MAR 18.5), kindly provided by Dr. Lewis Lanier,[12] was purified from culture supernatant by protein A-Sepharose chromatography. It was conjugated to allophycocyanine by Molecular Probes (Junction City, OR). Phycoerythrin-avidin conjugate was purchased from Becton-Dickinson (Monoclonal Center, Mountain View, CA).

Immunofluorescent staining

All staining was done in Hank's balanced salt solution (HBSS) containing 0.1% bovine serum albumin and 1 mM sodium azide. Single cell suspensions were treated with 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA to lyse erythrocytes. One million cells in 50 μl were incubated with a saturating amount of the desired antibody for 30 minutes at room temperature, and washed twice with HBSS before analysis. For three-color studies, 2×10^6 cells were stained and washed sequentially with unlabelled monoclonal B220-1; FITC-anti-mouse IgG_{2a}

(Southern Biotechnology Associates, Birmingham, AL). Normal mouse serum was added to block the anti-mouse IgG_{2a} followed by rat anti-mouse IgM (331.2); allophycocyanine-MAR 18.5; biotin conjugated anti-IgD (10.3-22); and phycoerythrin-avidin.

Flow cytometry

Analyses were performed with a modified FACS-II (Becton-Dickinson FACS Systems, Sunnyvale, CA) equipped with argon and krypton ion lasers and using logarithmic amplification. For three-color immunofluorescence, FITC and phycoerythrin were excited at 488 nm and allophycocyanine at 670 nm. FITC/phycoerythrin cross-channel compensation used a device constructed by T. Nozaki (Stanford University, Palo Alto, CA). Multiparameter data was collected in " list mode " and analyzed with a PDP 11/34 computer (Digital Equipment Corporation, Maynard, MA).

Cytotoxicity determination

The cytotoxic activity of the monoclonal antibodies was tested with a two-stage procedure: 6×10^7 spleen cells were incubated with a saturating amount of purified monoclonal antibody followed by appropriately diluted rabbit complement (Lo-Tox, Cedar Lane Laboratory, Hornby, ON, Canada) for 30 minutes at 37°C. Viable cells were recovered by differential flotation with lympholyte-M (Cedar Lane Laboratory) at 400 g for 20 minutes at 20°C.

Immunoprecipitation

NZB spleen cells were radiolabelled with ^{125}I catalyzed by lactoperoxidase.[13] The cells were lysed with 1% Nonidet NP-40 in 0.01 M sodium phosphate buffer pH 7.5. The solubilized membrane proteins were reacted with monoclonal antibodies and precipitiated with formalin-fixed *S. aureus*. The immune precipitates were washed three times, boiled in SDS sample buffer with 1% 2-mercaptoethanol and electrophoresed on 5-15 % gradient gels in the presence of sodium dodecyl sulfate. Molecular weight standards (45 to 200 kd) were from Bio-Rad, Richmond, CA. The gels were fixed, dried and autoradiographed.

RESULTS

B220-1 and B220-2 recognize B cell determinants

Two clones, termed B220-1 and B220-2, which originated in different wells, were isolated. Both were IgG_{2a} and bound well to Protein A at pH 8.2. The fraction of NZB thymus, bone marrow, spleen and lymph node cells stained by these and other anti-B cell monoclonal antibodies are shown in Table 1. B220-1, B220-2 and 14.8 labeled similar proportions of cells in the various lymphoid organs. The fractions stained by RA_3-3A1 was consistently less, presumbly because it is an efficiently aggregating IgM antibody. Thymocytes showed no staining. Dual parameter, fluorescence by light scatter, analysis of bone marrow showed that 70-80% of the positive cells were in the small cell fraction, the remainder being medium or large. The similar staining distribution of B220-1 and B220-2 with 14.8 and RA_3-3A1, which have been shown to identify

Table 1 Immunofluorescent staining of lymphoid organs

Cell suspension	Cells stained with*			
	B220-1	B220-2	RA3-3A1	14.8
Bone marrow	45.5	55.7	37.7	51.2
Spleen	45.9	42.5	32.6	48.1
Lymph node	16.8	18.7	14.6	27.7
Thymus	0	0	0	0

* Percentage of cells stained with directly fluoresceinated antibody.

Table 2 Cross blocking of binding by monoclonals B220-1 and B220-2

Blocking antibody	Fluorescent antibody*			
	B220-1	B220-2	RA3-3A1	14.8
B220-1	100.0	100.0	89.8	88.4
B220-2	75.0	100.0	48.7	44.1
RA3-3A1	52.2	40.9	100.0	ND+
14.8	61.5	62.0	ND	100.0

* NZB spleen cells were incubated with saturating amounts of the unlabeled blocking antibody for 20 minutes. The fluorescent antibody was then added, incubated for 30 minutes, washed and analyzed. The results are expressed as the percent inhibition of fluorescent antibody bound in comparison with the unblocked control.

\+ Not done.

B cells, suggested that these antibodies recognized primarily B cells and, in the bone marrow, might also include B cell precursors.

Despite the similar tissue distribution of B220-1 and B220-2, they appear to recognize different determinants, as shown by the cross-blocking experiments presented in Table 2. Each antibody is completely blocked by itself. However, B220-1 completely blocks binding of B220-2 but B220-2 only partially blocks the binding of B220-1. This suggests that the determinants recognized by these antibodies are spatially related but not identical epitopes on the same molecule.

The relationship between the presence of IgM and the determinant recognized by the monoclonals was examined by two-color immunofluoresence (Fig. 2a). Almost all IgM^+ cells were also $B220\text{-}1^+$ and $B220\text{-}2^+$. About 2% of IgM^- spleen cells expressed B220-1 and B220-2. Similar results were obtained in bone marrow cells.

The monoclonals RA_3-3A1 and 14.8, with staining patterns closely resembling B220-1 and B220-2, have been shown to react with all B cells as well as some pre-B cells and to recognize a 220,000 dalton form of T200, provisionally designated B220. To further characterize the cells bearing the antigen recognized by B220-1 and B220-2, we used two-color analysis to examine the correlation between these monoclonals and 14.8 and RA_3-3A1. Fig. 2b indicates close correlation between 14.8 and B220-1, while Fig. 2c shows a similar result for RA_3-3A1 and B220-1. However, binding of either B220-1 or B220-2 was not fully blocked by prior incubation of the cells with either 14.8 or RA_3-3A1 (Table 2) suggesting that the new antibodies bind to a different antigenic determinant.

Both monoclonals were cytotoxic: B220-1 killed 85% of B cells in comparison to 40% for B220-2.

B220-1 and B220-2 react with Lyt-2^+ peripheral T cells

Since 14.8, but not RA_3-3A1, gives weak staining of Lyt-2^+ peripheral T cells, two-color analysis was used to determine the behavior of the new monoclonals. Fig. 3 indicates that both B220-1 and B220-2, like 14.8 recognize Lyt-2^+ peripheral T cells.

Biochemical characterization

Immunoprecipitation of surface iodinated spleen cells showed that B220-1 and B220-2 recognized a molecule with an apparent molecular weight of 220 kd (Fig. 4). RA_3-3A1 and 14.8 also precipitated this 220 kd molecule, as expected.

Three-color immunoflourescence

One use of pan-B cell reagents is in three-color immunofluorescence. We used B220-1 to distinguish B cells from non-B cells, and derived the two-color IgM and IgD distribution for each population. As shown in Fig. 5, no $B220\text{-}1^-$ cells expressed surface immunoglobulin. The $B220\text{-}1^+$ cells show three regions in the IgM by IgD distribution: the predominant IgD bright, IgM dull population, as well as IgD dull, IgM bright group and an IgD bright, IgM bright subset.

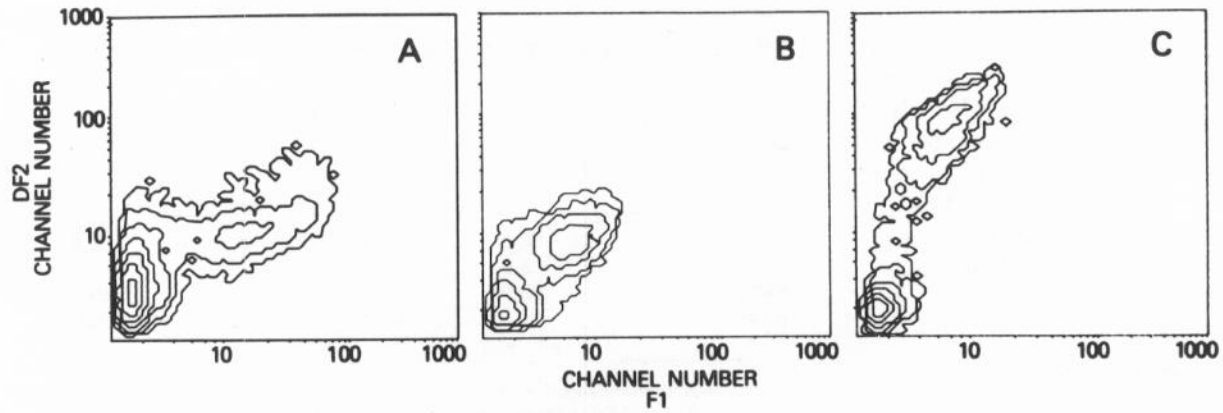

Fig 2 Contour plots of two-color immunofluorescence studies of NZB spleen cells. A FITC anti-IgM (green, X axis) and B220-1 (red, Y axis); B. FITC 14.8 (X axis) by B220-1 (Y axis); C. FITC B220-1 (X axis) by RA_3-3A1 (Y axis).

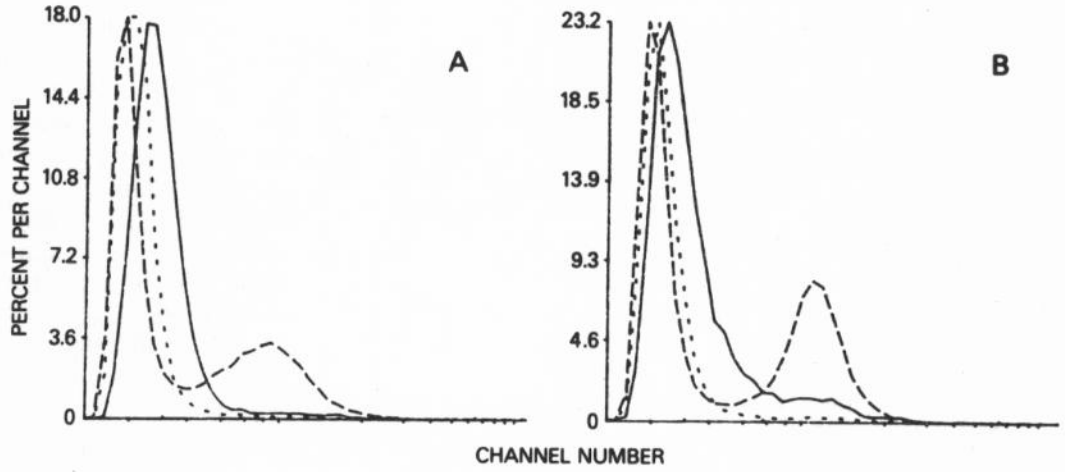

Fig. 3 Two color analysis of FITC B220-1 (A) or FITC 14.8 (B) with biotinylated anti-Lyt-2 or anti-L3T4/Texas Red avidin. To permit easier visualization of the contour results, "slices" were made to demonstrate green fluorescence corresponding to selected red fluorescence. The green fluorescences of Lyt-2$^+$ cells (solid) show weak staining in comparison with L3T4$^+$ T cells (dotted). The Lyt-2$^-$ cells gave bimodal distribution as expected.

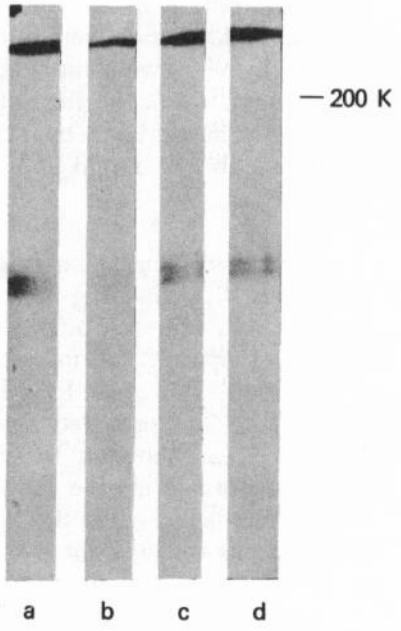

Fig. 4 Polyacrylamide gel electrophoresis of ^{125}I protein immunoprecipitates from NZB spleen. A. RA_3-3A1; B. 14.8; C. B220-1; D. B220-2. The band in the middle of the gel is immunoglobulin heavy chain.

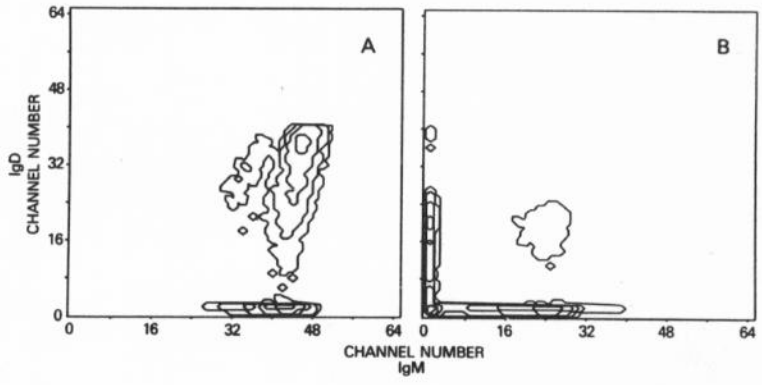

Fig 5 Three-color immunofluorescence analysis of IgM, IgD and B220-1. The correlated IgM (X axis) by IgD (Y axis) distribution derived from list mode data is presented for B220-1 positive (A) and negative (B) cells. A different logarithmic amplification system, which places negative cells in the lower third of the distribution and at the origin, was used in this experiment. Thus, the B220-1$^-$ cells are negative for IgM and IgD (B), while the B220-2$^+$ cells produce a three-lobed distribution for IgM and IgD (A).

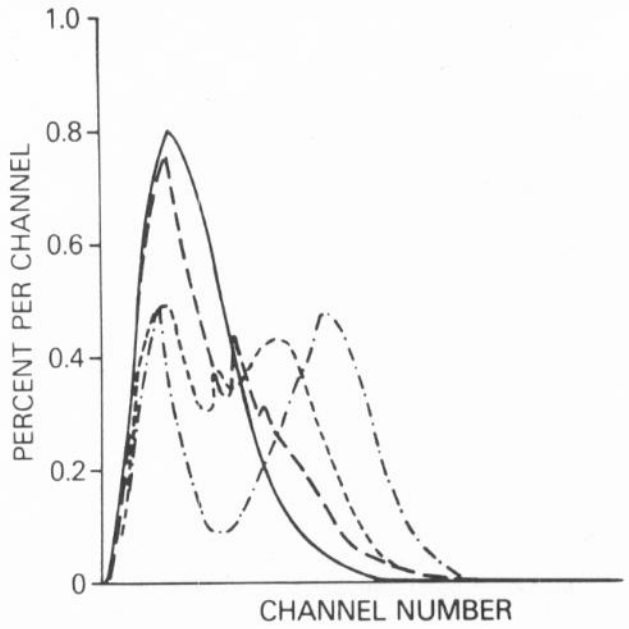

Fig 6 Fluorescence histograms of spleen cells stained by monoclonal B220-1, showing differences in staining intensity of BALB/c (···), NZNB (- - -), C57B1/6N (— —) and AKR (——).

Table 3 Strain distribution of B220-1 and B220-2

Strain	B220-1	B220-2
A/J	4*	4
AKR/J	1	2
AKR/N	1	1
AKR/J × DBA/2N	1	4
AL/N	4	3
BALB/CanN	4	4
CBA/J	4	4
CBA/N	3	4
CE/J	4	4
C3H/HeJ	4	3
C3H/SwSn	4	3
C3H/N	4	2
C57L/N	4	4
C57B1/6N	2	1
CBA/N × DBA/2N♂	3	4
CBA/N × DBA/2N♀	3	4
DBA/2N × CBA/N♂	3	3
DBA/2N × CBA/N♀	4	4
DBA/1N	1	4
DBA/2J	1	4
NFS	3	4
NZB	3	3
PC/J	4	3
Rf/J	4	4

*B cell staining was graded from 1 to 4.

Strain distribution

Spleen cells from a panel of mouse strains were tested for B220-1 and B220-2 reactivity. Interestingly, considerable variation in staining intensity was observed. Typical histograms are shown in Fig. 6 and the strain distribution data is presented in Table 3. B220-2, but not B220-1, is autoreactive since it recognizes AKR/J × DBA/2N F_1 B cells which were fused to generate the hybridoma. The data in Table 3 indicate that the DBA parent contributes this specificity to the F_1 hybrid.

DISCUSSION

Two monoclonal mouse IgG_{2a} antibodies which recognize B220, the B specific portion of the T200 molecule, were obtained from AKR/J × DBA/2N F_1 mice. These reagents react with cells in bone marrow, spleen and lymph node but not thymus. Two-color analysis indicates that 98% of the spleen cells that stained brightly by B220-1 or B220-2 also bear IgM and thus are B cells.

Rat anti-mouse monoclonal antibodies with similar specificities have been described. Coffman and Weissman reported that monoclonal antibodies, such as RA_3-3A1[3] and RA_3-2C2,[14] prepared against RAW 112, an Abelson leukemia virus-transformed murine cell line, bound to B cells and to sIg^- bone marrow cells which could give rise to B cells *in vitro*. The antigen recognized by this antibody had a molecular weight of 220 kd and the name B220 was suggested. Dessner and Loken[15] also described a rat monoclonal to the B lymphoma 70Z/3 which recognizes both normal B cells and cytoplasmic μ chain bearing cells. Kincade and coworkers produced the 14.8 monoclonal[9] which also bound to

all surface and cytoplasmic IgM bearing B cells. Although 14.8 did not react with thymocytes, it weakly stained peripheral Lyt-2^+ T cells, as do RA_3-2C2[16] and both of our antibodies.

Two-color immunofluorescence analysis and immunoprecipitation indicates that although B220-1 and B220-2 recognize the same molecule as 14.8 and RA_3-3A1, they recognize distinct determinants. This is shown by the inability of 14.8 or RA_3-3A1 to block the binding of B220-1 or B220-2. Staining of $Ly2^+$ peripheral T cells suggest that these monoclonals are more closely related to 14.8 than to RA_3-3A1. Furthermore, the epitopes recognized by B220-1 and B220-2 are also distinct. B220-1 binds to most, if not all, of the molecules recognized by B220-2, as evident by the crossblocking experiment (Table 2). This led us to perform the strain survey shown in Table 3. The differential expression of B220 antigen on various strains of mice indicates polymorphism in the B220 antigen system.

The B220-1 antibody is useful for cytotoxic elimination procedures since it is directly cytotoxic, in contrast to 14.8. In the past, panning has been utilized for elimination of B cells but yields have been very poor, therefore, these monoclonals could be utilized for complement mediated cytotoxicity. It has been established that the Ly-5 alloantisera define a polymorphic determinant of T_{200} glycoprotein. Our results suggest that B220 also exists in polymorphic form. Whether this is due to variation in carbohydrate content or in the primary amino acid seqence of the B220 molecule is not yet known.

In comparison to T lymphocyte differentiation, B cell differentiation pathways are not well defined. With the emergence of three color immunofluorescence and monoclonal antibodies of defined specificities it should be possible to define the differentiation pathways. One of the problems with three color immunofluoresence is the cross reactivity of various reagents used. Since these monoclonals are of mouse origin, they can be directly labelled and used in such experiments without interacting with rat antibodies.[17]

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