B-CELL SUBPOPULATIONS IDENTIFIABLE BY TWO-COLOR FLUORESCENCE ANALYSIS USING A DUAL-LASER FACS*

R. R. Hardy, K. Hayakawa, J. Haaijman,† and L. A. Herzenberg

Department of Genetics
Stanford University School of Medicine
Stanford, California 94305

†*Institute of Experimental Gerontology*
Health Organization TNO
Rijswijk, the Netherlands

Two-color immunofluorescence studies using monoclonal antibodies specific for IgM[1] and IgD,[2] labeled respectively with fluorescein and "Texas Red" (a new red-fluorescent dye), reveal several previously unrecognized B-cell subpopulations in spleen and lymph node. Measured individually, these surface markers (IgM and IgD) show only that B-cells are broadly heterogenous with respect to the amount of surface Ig expressed; however, measured simultaneously with a dual-laser Fluorescence Activated Cell Sorter (FACS) the correlated expression of these B-cell surface markers defines three B-cell subpopulations.

FIGURE 1 compares two displays of the IgM-IgD staining pattern for spleen cells from a one month old NZB/J mouse. Panel (a) shows a linear-linear display of the data while panel (b) presents data from the same cells analyzed instead with logarithmic amplifiers. The log-log display shows clearly that the brightest IgM^+ cells include many IgD^+ cells and that the bright IgD^+ population is IgM^+. For the linear illustration if we chose a gain for Texas Red low enough to keep most of the brighter IgD^+ cells on scale, then the majority of IgM^+ cells appear to be IgD^-, whereas at a higher linear gain (not illustrated) many of these can be seen to be IgD^+. Similarly, if the fluorescein-IgM gain is chosen so as to keep most of the IgM^+ cells on scale, then this gives the impression of a large IgD^+, IgM^- population which again is an artifact of the particular linear gain settings employed. Careful analysis by specialists of data taken at several pairs of linear gains would be required to reach conclusions that are obvious to the uninitiated from the log-log display.

The IgM-IgD subpopulations defined by our studies are diagrammed schematically in FIGURE 2a. One of these populations (I), which is predominant in spleen and constitutes the overwhelming majority of B-cells in lymph nodes, is missing in CBA/N (Xid) mice known to be deficient with respect to their B-cell immune responses.[3] This population, which is relatively low in IgM and intermediate in IgD, clearly shows a positively correlated expression of these markers. The other population is high in IgM but quite heterogeneous with respect to IgD levels. It appears that this latter population is further divided into bright and dull IgD subpopulations (II and III) on the basis of differences between spleen and lymph nodes.

We have carried out an extensive IgM-IgD staining survey of mouse lymphoid cell populations from different strains, ages, and organs. We will describe the

*This work was supported in part by NIH Grants AI-01287, CA-04681 and GM-17367.

0077-8923/82/0399-0112 $01.75/0

studies in detail elsewhere; however, the highlights are summarized in FIGURES 2, 3 and 4. FIGURE 3 presents the organ distribution of the three subpopulations and simultaneously illustrates the CBA/N defect. FIGURE 4 shows that population I is the last subpopulation to appear during ontogeny in the spleen. Finally, FIGURE 2 (b-f) demonstrates that there are genetically controlled differences in the mature levels of population II with BALB/c showing the low phenotype and CBA (characteristic of the high) showing approximately twice the number in this subpopulation. Organ and strain variation of the three populations are summarized in TABLE 1.

Two-color staining with monoclonal antibodies specific for ThB[4] and for a subset of splenic B-cells has yielded further information regarding the subpopula-

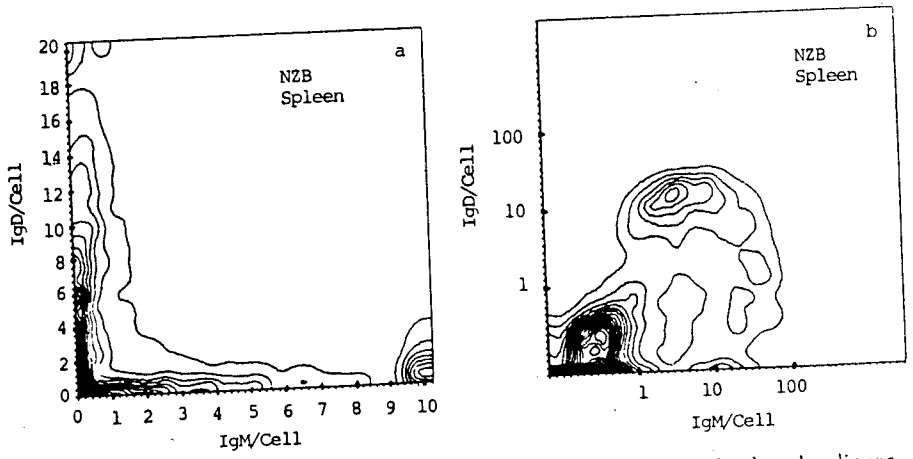

FIGURE 1. A comparison of IgM-IgD two-color immunofluorescence displayed as linear-linear (a) or log-log (b) contour plots. One month NZB/J spleen cells (10^6) were stained with 0.5 micrograms of fluorescein-labeled monoclonal rat anti-IgM[1] and 0.5 micrograms of biotinated monoclonal mouse anti-IgD[2] in 100 microliters of biotin-free RPMI-1640 containing 10 mM HEPES buffer, 0.1% sodium azide, and 3% newborn calf serum for 30 minutes of 0° C. Cells were washed three times with RPMI and stained with one microgram of Texas Red-avidin in 50 microliters of RPMI for 30 minutes at 0° C. Cells were washed three times with RPMI, resuspended in 300 microliters of the same buffer and analyzed on the dual-laser FACS equipped with both linear and logarithmic amplifiers. To permit subsequent analyses, "list-mode" data recording the scatter and two fluorescence measurements for each cell were collected on 30,000 cells using a VAX 11/780 computer.

tions described above. Previous single parameter staining with the monoclonal anti-ThB divided mouse strains into bright (or "high") and dull (or "low") for this marker.[5] Furthermore, ThB expression on B-cells was shown to be under autosomal co-dominant control so that an F1 hybrid of a "high" and a "low" was "intermediate." Two parameter staining of ThB and IgM illustrated in FIGURE 5 (a and b) shows that most of this variation occurs in the IgM dull population (I) with the IgM bright populations remaining uniformly high for ThB. Another monoclonal antibody previously shown to stain spleen cells weakly ("E2")[6] stains only the high IgM populations (II and III) as shown in FIGURE 5 (c and d).

FIGURE 6 presents Ia expression on these populations which was examined

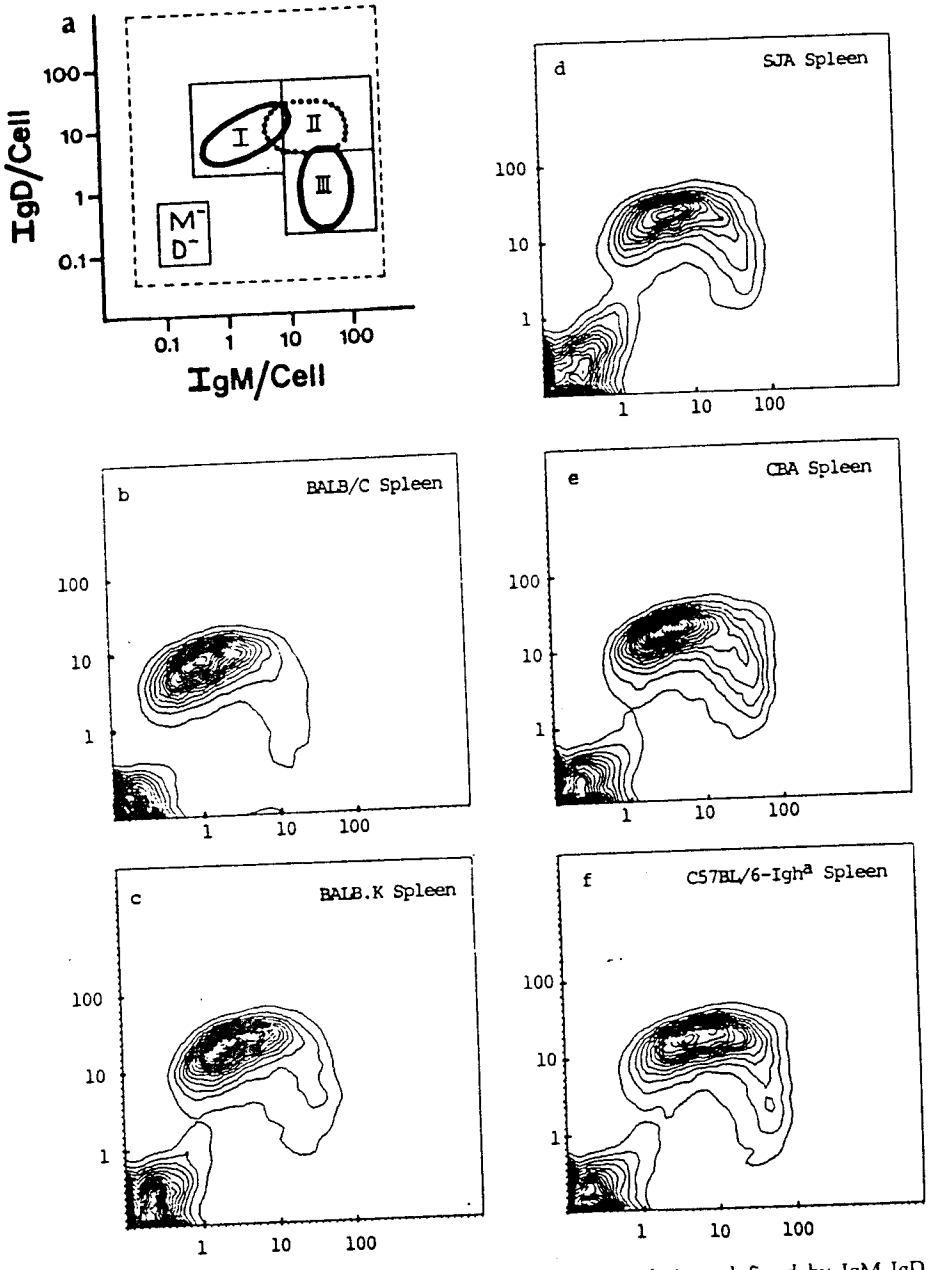

FIGURE 2. (a) A schematic diagram of the B-cell subpopulations defined by IgM-IgD immunofluorescence staining. (b-f) There are two levels at which population II occurs depending on strain. BALB/c and BALB.K are low (b, c), while SJA, CBA and C57BL/6 are high (d-f).

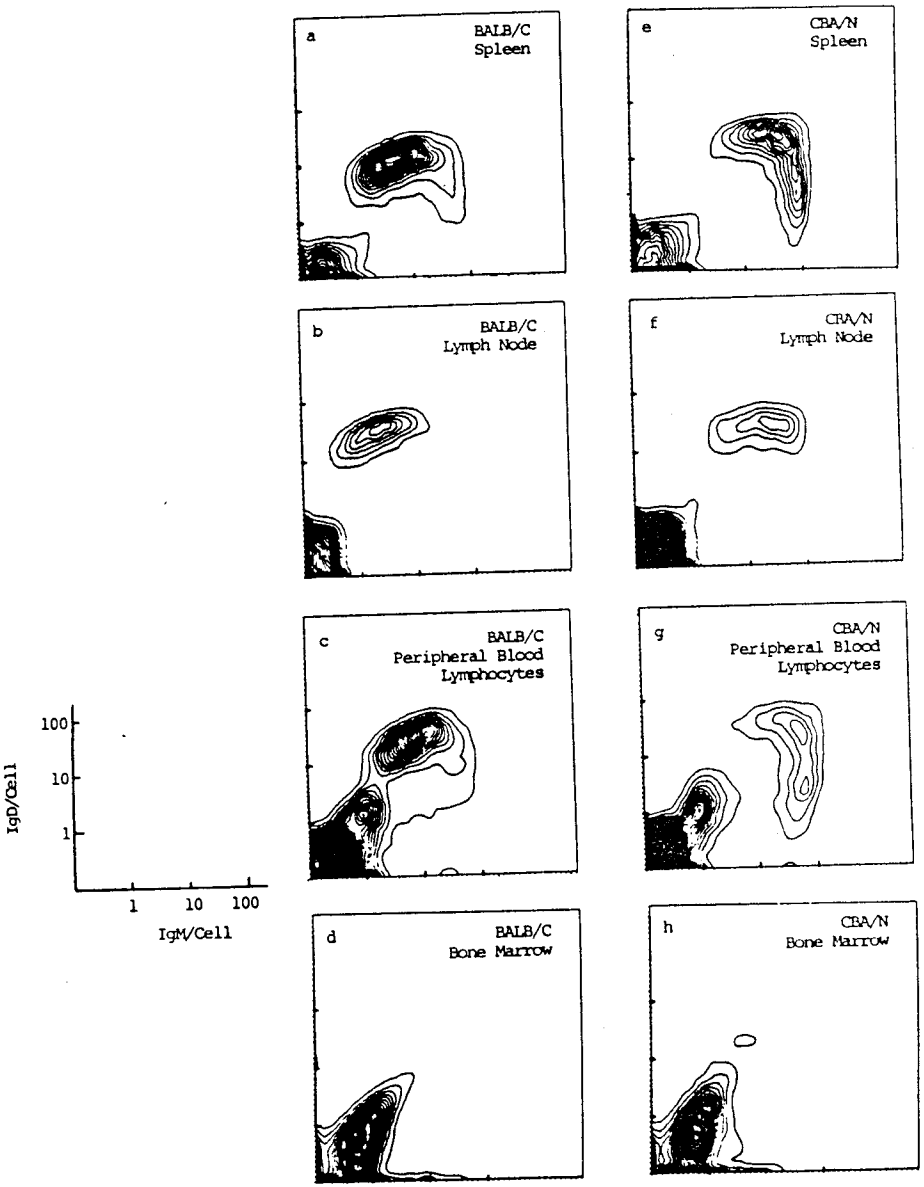

FIGURE 3. Variation of the three populations with lymphoid organ both in normal (BALB/c) and defective (CBA/N) mice. Spleen, lymph node, peripheral blood lymphocytes and bone marrow of BALB/c: a-d, of CBA/N: e-h.

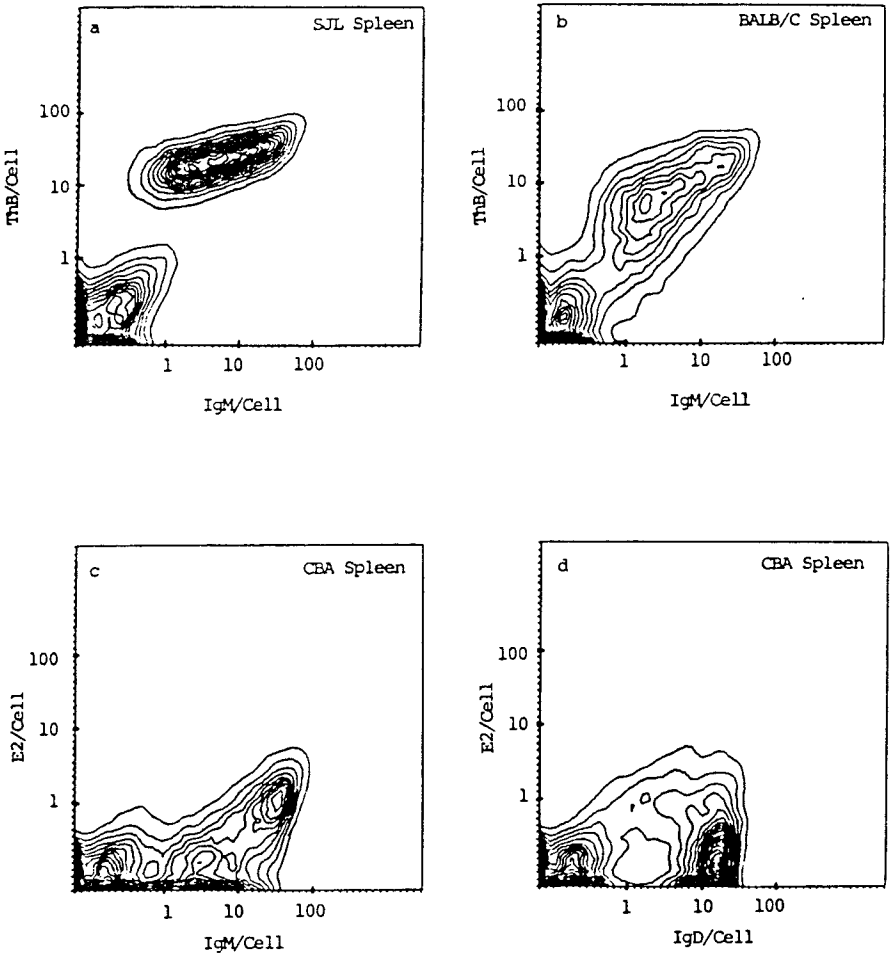

FIGURE 5. (a-b) The major differences between ThB high and low strains is due to a difference in surface density on population I. SJL is a high strain (a) and BALB/c is a low strain (b). (c-d) E2 is a surface antigen found only on populations II and III; that is, it is found on all bright-IgM cells (c), but on IgD cells with widely varying surface density (d).

lymphomas expressing Lyt-1 and IgM.[12] Two-color staining of a number of different mouse strains consistently showed 1-2% of spleen cells with this phenotype, but the most impressive demonstration of its presence is in NZB mice which have 5-10%. These mice also have an unusual IgM/IgD profile with many more population III cells and many fewer population II cells compared to similar high-IgM strains. The cells lack Thy-1 and Lyt-2 which is consistent with the phenotype described in the *in vitro* assay (K. Okumura, K. Hayakawa and T. Tada, manuscript in preparation). Whatever role these cells might play in the well-known autoimmune disorder of NZB mice[13] is speculative at present and can be investigated using sorted cells.

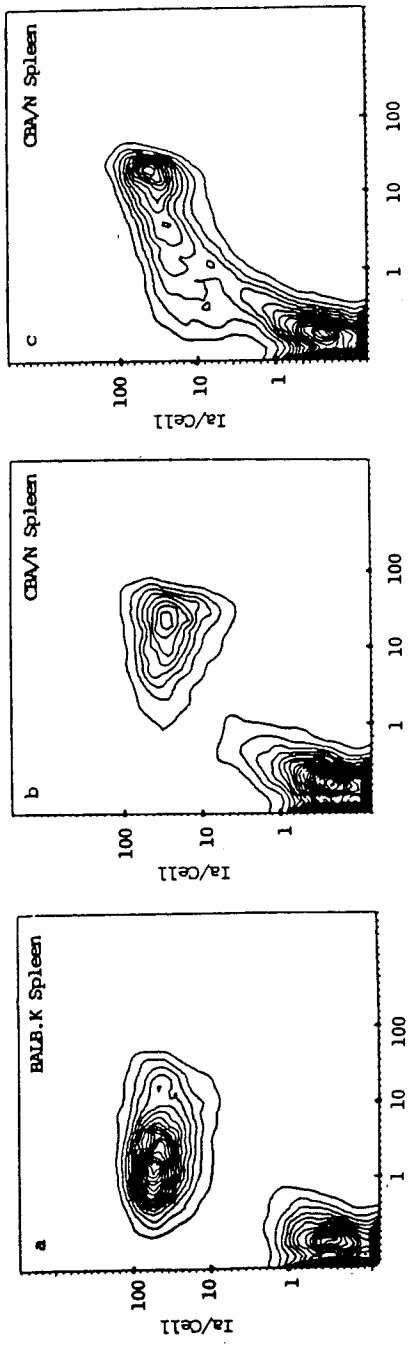

FIGURE 6. Ia expression is lower on population III. This is not obvious in the BALB.K staining (a) where population I dominates, but is shown more clearly by staining CBA/N spleen. The IgM-Ia staining (b) shows that the lowest Ia cells are IgM-bright, while the IgD-Ia staining (c) shows the lowest Ia cells are IgD-dull. Together these stains define population III.

using a monoclonal antibody specific for Ia of k, specificity 2.[2] Staining Balb.K spleen showed only slight variation of Ia density among the three populations with greater heterogeneity in the high-IgM populations. Staining CBA/N spleen simplified the situation by eliminating population I (high-Ia) and clearly showed that population III expresses reduced amounts of Ia compared to the other two

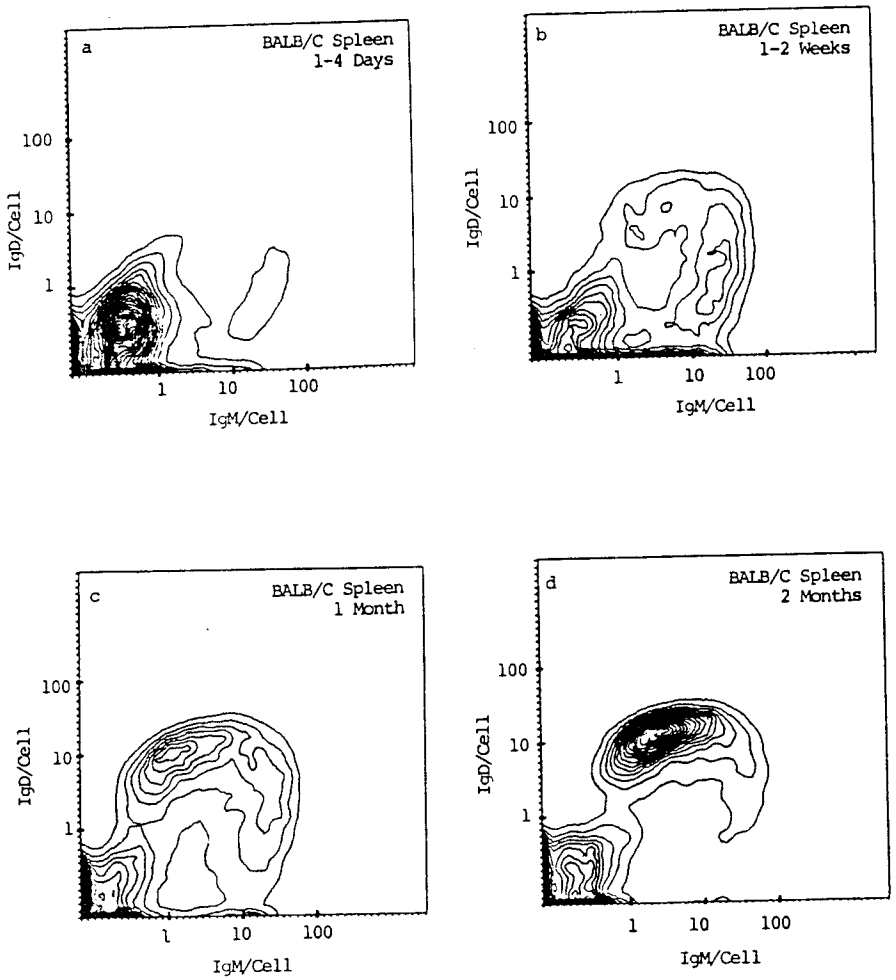

FIGURE 4. Population I appears late in spleen ontogeny. BALB/c spleen: (a) 1-4 days; (b) 1-2 weeks; (c) 1 month; (d) 2 months or older.

populations. The relative expression of several surface antigens on the three populations is summarized in TABLE 2.

Functional distinctions of these three subpopulations are at present unknown, but the immune defects of CBA/N mice certainly suggest that they may be functionally distinguishable. CBA/N mice (which lack population I) have low

TABLE 1

PERCENTAGES OF CELLS IN IgM/IgD-DEFINED B-CELL SUBPOPULATIONS*

Strain	Organ	I	II	III
BALB/c	Spleen	40	8	2
	Lymph node	20	2	<1
CBA	Spleen	30	17	3
	Lymph node	18	5	<1
CBA/N	Spleen	4†	16	8
	Lymph node	<1	4	<1
NZB/N	Spleen	27	9	7
	Lymph node	15	2	<1

*CBA/N mice lack the major B-cell subpopulation found in spleen and lymph nodes in normal mice. NZB mice, on the other hand have an elevated level of population III compared to normal mice. Data show the percentages of total spleen or lymph node cells present in the B-cell subpopulations shown in FIGURE 2a. Percentages for populations I and II may be in error by as much as five percent in the BALB/c, CBA and NZB determinations due to overlap between these populations. Subpopulations defined by 2-color FACS analysis shown in FIGURE 2.

†This figure (4%) is attributable to overlap from population II.

serum levels of IgM and IgG3,[7] have reduced numbers of splenic B-cells,[8] are unable to respond to type II thymus independent antigens (eg., TNP-ficoll),[9] and lack a B-cell surface antigen known as Lyb-3.[10] It is tempting to speculate that these deficiencies are all due to the lack of population I, however preliminary data indicate that the CBA/N B-cells in populations II and III are defective in some respects compared to cells in these populations from normal mice. This points out a clear danger in considering CBA/N B-cells as normal CBA B-cells minus a subpopulation of cells.

Population III is very unusual because at least some cells in this subpopulation express the T-cell antigen Lyt-1 (see FIGURE 1). One of us (K.H.) previously detected a cell with this phenotype (Ig+,Lyt-1+) in an *in vitro* antibody formation assay[11] and previous single fluorescence staining has demonstrated mouse B-

TABLE 2

LEVEL OF EXPRESSION OF SEVERAL CELL SURFACE ANTIGENS ON THE IgM-IgD DEFINED SUBPOPULATIONS*

Surface Marker	I	II	III
IgM	1-50	100	100
IgD	50-100	100	10
Ia	100	100	50
ThB	1-50;50‡	100	100
E2	<10	100	100
Lyt-1†	<1	<1	10

*The three populations are quite heterogenous in their surface phenotypes for a number of cell surface antigens. The surface densities are normalized separately for each antigen.

†Density relative to average Lyt-1 density on T-cells.

‡Depending on ThB(low) or ThB(high) strain.

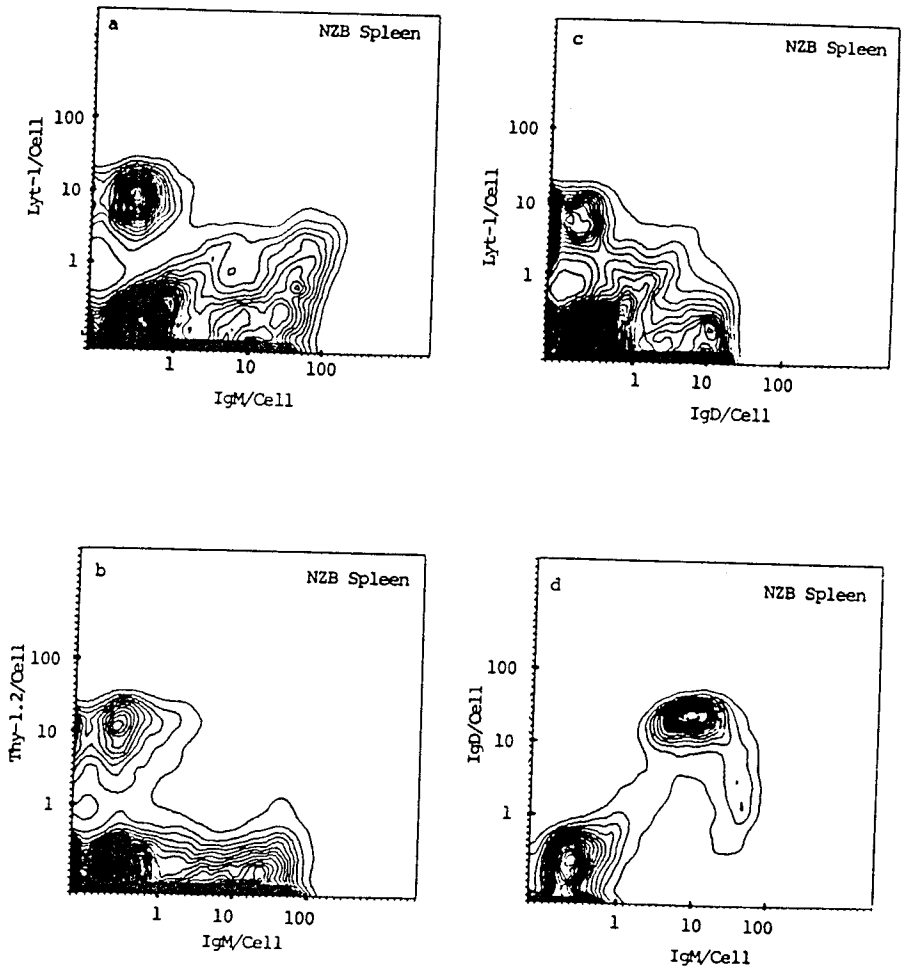

FIGURE 7. The NZB mouse spleen contains elevated amounts of a cell expressing both IgM and the T cell antigen Lyt-1 (a). These cells do not express Thy-1 (b), but do express low amounts of IgD (c). This places the cells in the IgM-IgD pattern as population III and indeed the IgM-IgD staining profile(d) is unusual with increased population III and decreased population II compared to other strains tested.

ACKNOWLEDGMENTS

We acknowledge the Institute of Experimental Gerontology, Health Organization TNO, Rijswijk, the Netherlands, for providing the CBA/N mice used in these studies. We thank Dr. P. Kincade for the monoclonal rat anti-IgM, Dr. D. Parks for invaluable assistance with the dual-laser FACS and Mr. W. Moore for writing the VAX data handling programs.

REFERENCES

1. KINCADE, P. W., G. LEE, L. SUN & T. WATANABE. 1981. J. Immunol. Meth. **42:** 17-26.
2. OI, V. T., P. P. JONES, J. W. GODING, L. A. HERZENBERG & L. A. HERZENBERG. 1978. Curr. Top. Microbiol. Immunol. **81:** 115-129.
3. SCHER, I., M. M. FRANTZ & A. D. STEINBERG. 1973. J. Immunol. **110:** 1396-1401.
4. YUTOKU, M., A. L. GROSSBERG & D. PRESSMAN. 1974. J. Immunol. **112:** 1774-1781.
5. ECKHARDT, L. A. & L. A. HERZENBERG. 1980. Immunogenetics **11:** 275-291.
6. LEDBETTER, J. A. & L. A. HERZENBERG. 1979. Immunol. Rev. **47:** 63-90.
7. PERLMUTTER, R. M., M. NAHM, K. E. STEIN, J. SLACK, I. ZITRON & W. E. PAUL. 1979. J. Exp. Med. **149:** 993-998.
8. FINKELMAN, F. D., A. H. SMITH, I. SCHER & W. E. PAUL. 1975. J. Exp. Med. **142:** 1316-1321.
9. MOSIER, D. E., J. J. MOND & E. A. GOLDINGS. 1977. J. Immunol. **119:** 1874-1878.
10. HUBER, B., R. K. GERSHON & H. CANTOR. 1977. J. Exp. Med. **145:** 10-20.
11. OKUMURA, K., K. HAYAKAWA & T. TADA. J. Exp. Med. In press.
12. LANIER, L. L., N. L. WARNER, J. A. LEDBETTER & L. A. HERZENBERG. 1981. J. Immunol. **127:** 1691-1691.
13. WARNER, N. 1979. *In* Autoimmunity, N. Talal, Ed.: 33-62. Academic Press. New York, N.Y.