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B-cell subpopulations identified by two-colour fluorescence analysis

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The simultaneous and rapid measurement of the amounts of two different fluorochrome-coupled antibodies bound to single cells (two-colour immunofluorescence) provides a very powerful means for the identification of lymphocyte subpopulations[1]. Using a dual-laser fluorescence-activated cell sorter (FACS) we show that two monoclonal antibodies, anti-IgM and anti-IgD, labelled respectively with fluorescein and 'Texas red' (a new red-fluorescent dye) reveal several previously unrecognized B-cell subpopulations in mouse spleen and lymph nodes. Measured individually, these surface markers (IgM and IgD) show only that B cells are broadly heterogeneous with respect to the amount of surface immunoglobulin expressed[2]; however, measured simultaneously, they clearly define at least two B-cell subsets. One of these populations, 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].

The B-cell populations in BALB/c spleen (Figs 1, 2) are resolved into two subsets on the basis of the amount of IgM expressed. The predominant population (labelled I in the diagram), which has relatively little surface IgM, expresses intermediate to high levels of surface IgD and constitutes ~60% of the splenic B cells (Table 1). This population is even more predominant in BALB/c lymph nodes where it constitutes >80% of the B cells.

The remaining BALB/c splenic population(s), which express 10–100 times more IgM, appear to be further subdivided according to the amount of surface IgD expressed (labelled II and III) and the organ localization of the cells. The 'high-IgD, high-IgM' population (II) constitutes ~20% of the B cells in spleen and lymph nodes. The 'low-IgD' population (III) is distinguishable more by its apparent absence from lymph nodes (see Fig. 1) than by any clear-cut demarcation in the staining pattern.

Low-angle light scatter measurements in the FACS, which provide an index of cell size[4], further distinguish these populations. Population I is the major stained component of the smaller (by scatter) cells; population II is found predominantly among the larger cells; and population III is found amongst cells in an intermediate size range. This scatter difference is another parameter which should help in isolating these subpopulations for use in functional assays.

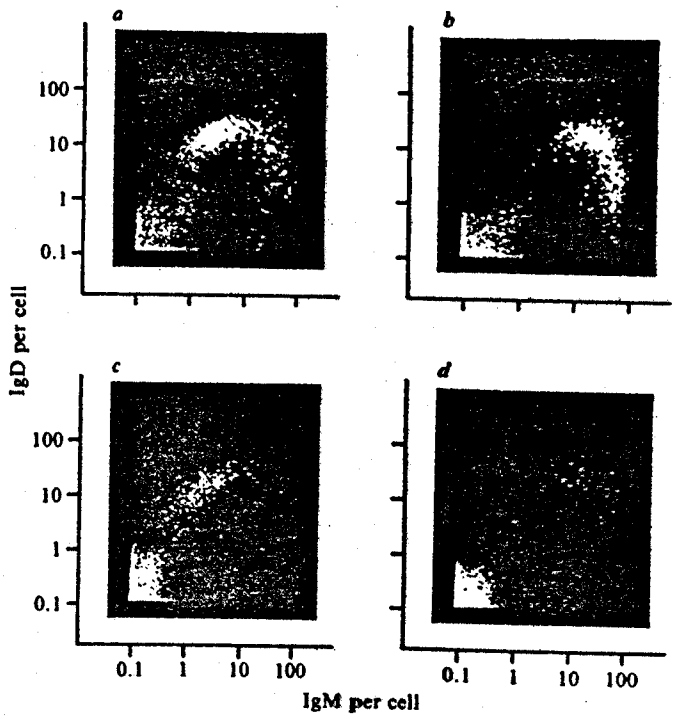

Fig. 1 B-cell subpopulations defined by two-colour FACS analysis of cells stained simultaneously with anti-IgM and anti-IgD antibodies. The individual points in the 'dot plots'[14] shown represent the amounts of IgD and IgM expressed on 3,000 individual cells from spleen and lymph nodes in CBA/N and BALB/c mice. The relative amounts of IgM and IgD per cell are indicated on the axes. *a*, BALB/c spleen; *b*, CBA/N spleen; *c*, BALB/c peripheral lymph nodes; *d*, CBA/N peripheral lymph nodes. Spleen or lymph node cells (10^6) were stained with 0.5 μg of fluorescein-labelled monoclonal rat anti-IgM[15] and 0.5 μg of biotinated monoclonal mouse anti-IgD[16] antibodies in 100 μl of biotin-free RPMI-1640 containing 10 mM HEPES buffer, 0.1% sodium azide and 3% newborn calf serum for 30 min at 0 °C. Cells were washed three times with RPMI and stained with 1 μg of Texas red–avidin in 50 μl of RPMI for 30 min at 0 °C. Cells were washed three times with RPMI, resuspended in 300 μl of the same buffer and analysed on the dual-laser FACS equipped with 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.

Studies with CBA/N mice further clarify the definition of the B-cell subpopulations. These mice, which carry an X-linked B-cell defect, have almost no detectable population I cells in lymph nodes or spleen. Their splenic B cells, which consist entirely of high-IgM populations, are divided between cells comparable with the cells in populations II and III in BALB/c spleen. The CBA/N spleen also has many more IgM-, IgD- double negative cells than the BALB/c spleen. As might be expected, CBA/N spleen cells have the same scatter correlations as BALB/c spleen cells, except that the small cells lack both IgM and IgD.

There are only a few immunoglobulin-positive cells in CBA/N lymph nodes and these stain exclusively in the region of population II. Thus, when population I is absent from lymph node and consequently does not obscure the analysis of the minor B-cell populations present, populations II and III are distinguishable by their locations in the IgM, IgD fluorescence plots.

Thus the combined data from two-colour FACS analyses of CBA/N and BALB/c spleen and lymph node demonstrate that the heterogeneous expression of IgM and IgD on the whole B-cell population reflects three (or more) subpopulations, each with characteristic levels of surface immunoglobulins. Studies of the appearance of these populations during development, and the disappearance of the B-cell population(s) present predominantly during neonatal life, indicate the presence of at

Table 1 Per cent of cells in IgM/IgD defined B-cell subpopulations

Strain	Organ	I	II	III
BALB/c	Spleen	30	10	10
	Lymph node	17	4	<1
CBA/N	Spleen	<4*	20	15
	Lymph node	<1	4	<1
CBA	Spleen	23	18	8
	Lymph node	10	4	<1

CBA/N mice lack the major B-cell subpopulation found in spleen and lymph nodes in normal mice. Subpopulations were defined by two-colour FACS analysis (Fig. 2). Data show the per cent of total spleen or lymph node cells present in the B-cell subpopulations shown in Fig. 2. Percentages for populations I and II may be in error by as much as 5% in the BALB/c and CBA determinations due to overlap between these populations.

*This value (4%) is attributable to overlap from population II.

least one more B-cell subpopulation characterized by the lack of surface IgD (J.H., R.R.H., K.H. and L.A.H. in preparation). While it is clear that population I reaches mature level last[5] the relationship between these populations is currently unknown. An allotype-specific IgM–IgD stain carried out on sorted cells transferred to allotype congenic mice should help to clarify these relationships.

The absence of population I in CBA/N mice fits with several previous observations: the higher IgM/IgD ratio on B cells from CBA/N spleen[6] arises from a lack of the low IgM/IgD ratio (population I) cells in these mice; the absence of certain B cell determinants [Lyb3 (ref. 7), Lyb5 (ref. 8), Lyb7 (ref. 9)] in CBA/N is apparently due to the presence of these markers exclusively on population I cells; the presence of one of these markers (Lyb3) on virtually all normal lymph node B cells, but on only a fraction of normal spleenic B cells[10], is consistent with the relative amounts of population I in the two organs.

CBA/N mice have an extensively studied immune deficiency: they do not make antibody to type II thymus-independent antigens (such as dinitrophenyl–Ficoll)[11]; their spleen cells fail to give the usual *in vitro* proliferative responses shown by normal spleen cells stimulated with anti-immunoglobulin antisera[12]; and their serum IgM and IgG3 levels are very low compared with normal mice[13]. We speculate that the B cells responding to dinitrophenyl–Ficoll or to anti-immunoglobulin antisera in the proliferative assay are contained in subpopulation I (missing in CBA/N). The B cells responsible for a large part of IgM and IgG3 antibody production also may be included in this subset. We should be able to test these hypotheses and assign functions to the B-cell populations defined here by

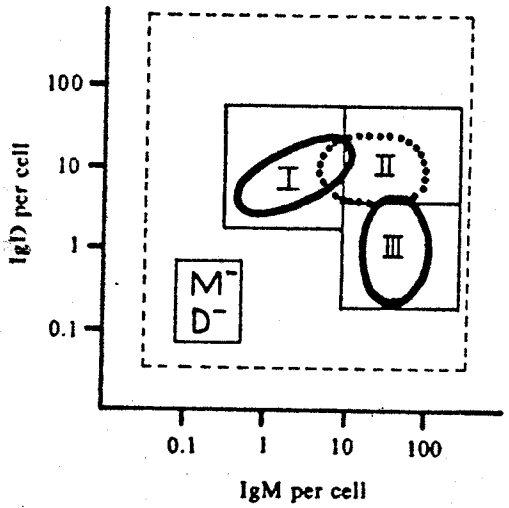

Fig. 2 Diagram of the B-cell subpopulations shown in Fig. 1 (see text). The integration bounds used to obtain the percentages in Table 1 are also shown.

sorting these populations before appropriate *in vitro* and adoptive transfer assays.

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