© Macmillan Journals Ltd., 1983

Demonstration of B-cell maturation in X-linked immunodeficient mice by simultaneous three-colour immunofluorescence

R. R. Hardy, K. Hayakawa, D. R. Parks & L. A. Herzenberg

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

CBA/N mice carrying the X-linked immune deficiency gene (*xid*) have fewer splenic B cells than normal CBA mice and are unresponsive to a certain class of antigens[1]. Studies of B-cell surface-marker expression[2] and immune responsiveness[3] have led to the commonly accepted idea that the B cells in adult *xid* mice are immature and resemble the B cells of young (1–3 week old) normal mice. That is, like young animals, *xid* mice lack cells in the most numerous of three IgM/IgD B-cell subpopulations (designated I in Fig. 1*a*, *b*) present in adult spleen[4,5]. We now report, however, that this picture is an oversimplification and that in fact the B cells in adult *xid* mice differ from those present in either adult or young normal mice. Using quantitative three-colour fluorescence-activated cell sorter (FACS) analyses, we have compared the correlated expression of IgM, IgD and a newly discovered B-lymphocyte antigen (BLA-1) on splenic B cells in normal and *xid* mice. We show here (1) that most B cells in adult *xid* mice (as in normals) are BLA-1$^-$ whereas all B cells in young animals are BLA-1$^+$; (2) that the major difference in the IgM/IgD B-cell subpopulations found between *xid* and normal mice is limited to the BLA-1$^-$ cells; and (3) that *xid* mice have increased numbers of BLA-1$^+$ population III B cells.

All IgM-bearing cells in the spleens of 2-week-old animals express BLA-1 (recognized by rat monoclonal antibody 53-10.1)[6], whereas less than half of the IgM-bearing spleen cells in adults carry this antigen (Fig. 2). BLA-1 therefore appears to be absent from the late-developing more 'mature' population of B cells that normally predominates in adult animals. The BLA-1 antigen is also found on IgM$^-$ cells, particularly in the spleens of young mice (Fig. 2) and in adult bone marrow (data not shown); however, it is absent from Thy-1$^-$ cells in spleen or thymus (data not shown) and thus among lymphocytes is expressed only on a subpopulation of B cells (in preparation).

The decreased expression of BLA-1 on 'mature' B cells introduces a new approach for testing the validity of the current paradigm of B-cell development: that the B cells in CBA/N mice are a 'less mature' population of normal B cells and that the inability of these cells to respond to certain antigens is a result of this 'immaturity'[3]. B cells in adult CBA/N mice have been considered to be less mature because they lack a family of serologically defined B-cell antigens (Lyb-3, 5, 7) which are missing from all B cells in normal neonatal animals, but are present on most B cells in the normal adult[7–9]. If it is correct that 'mature' B cells fail to develop in *xid* mice, then all B cells in adult *xid* mice should express BLA-1 since this antigen is a marker of immature B cells. We find this not to be the case.

Two-colour analyses of normal and *xid* spleen cells for the correlated expression of IgM and BLA-1 show that whereas all splenic B cells in young normal and *xid* mice bear BLA-1, a considerable portion of B cells in adult spleens from both types of mouse do not express this antigen (Table 1). Thus, rather than splenic B cells of adult *xid* mice resembling B cells from young *xid* (or normal) mice, they in fact (on the basis of BLA-1 expression) are more similar to normal adult B cells even though they lack the major population of late-arising low-IgM high-IgD B cells present in normal mice (population I).

To resolve the apparent contradiction between the similarity of B cells in normal and *xid* adult animals (with respect to BLA-1 expression) and the previous demonstration that the presumably mature B cells in population I are missing from *xid* spleen, we have developed three-colour immunofluorescence techniques which allow measurement of the correlated cellular expression of BLA-1, IgM and IgD. Previous work[10] described the use of a dye protein (called phycoerythrin, PE) derived from certain strains of algae to obtain good-quality two-colour immunofluorescence from a single laser exciting the fluorescence of fluorescein and PE at 488 nm. Electronic compensation[11] for the small overlaps of fluorescein fluorescence on the PE detector and PE fluorescence on the fluorescein detector yield signals essentially identical to those with either reagent used alone. We have modified our standard two-laser/two-colour setup[12] to allow use of the single-laser/two-colour system in conjunction with the second laser (retuned to 615 nm) to excite a third immunofluorescence from the dye protein, allophycocyanin (APC). This system will be described in detail elsewhere (in preparation).

As expected from two-colour analyses with IgM and BLA-1, three-colour studies with IgM, IgD and BLA-1 show that all splenic B (IgM-bearing) cells are BLA-1 positive in both normal

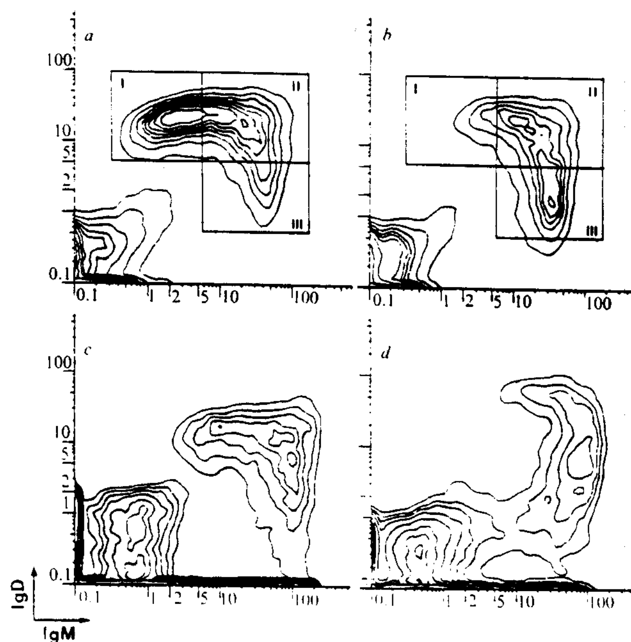

Fig. 1 CBA/N mice lack a population of B cells characterized by high levels of IgD and intermediate to low levels of IgM (two-colour analyses). IgM/IgD-stained spleen cells are shown from: *a*, CBA, 3 months old; *b*, CBA/N, 3 months old; *c*, CBA, 2 weeks old; and *d*, CBA/N, 2 weeks old. The boundaries of the three previously described B-cell populations[4,5] are drawn on the contour plots.

Methods: Spleen cells from which the erythrocytes had been lysed by 0.165 M ammonium chloride were stained in a two-step protocol in microtitre wells as described previously[14]. Green fluorescence: fluorescein-conjugated rat monoclonal anti-IgM[19] in the first incubation. Red fluorescence: biotinated mouse monoclonal anti-IgD (specific for the *a* allotype)[20] in the first incubation followed by Texas-red-labelled avidin in the second incubation. Stained cells were analysed on a dual-laser fluorescence activated cell sorter FACS equipped with logarithmic amplifiers (for the fluorescence channels) to measure light scatter (size) and the amounts of sets of fluorochrome-labelled monoclonal reagents bound to individual cells[12]. Individual measurements on 30,000 (live) cells were collected and stored as list mode data on a VAX-11/780 computer for later analysis. Data are presented as contour plots that can be viewed as representations of three-dimensional surfaces in which the levels of green and red fluorescence per cell define the location of cells on a (64×64) grid and the frequency of cells at each location defines the elevation at that location. After smoothing this surface, contour lines are drawn such that equal numbers of cells (typically 10% of the total) fall between each pair of adjacent contour lines. Regions with more contours thus have greater numbers of cells.

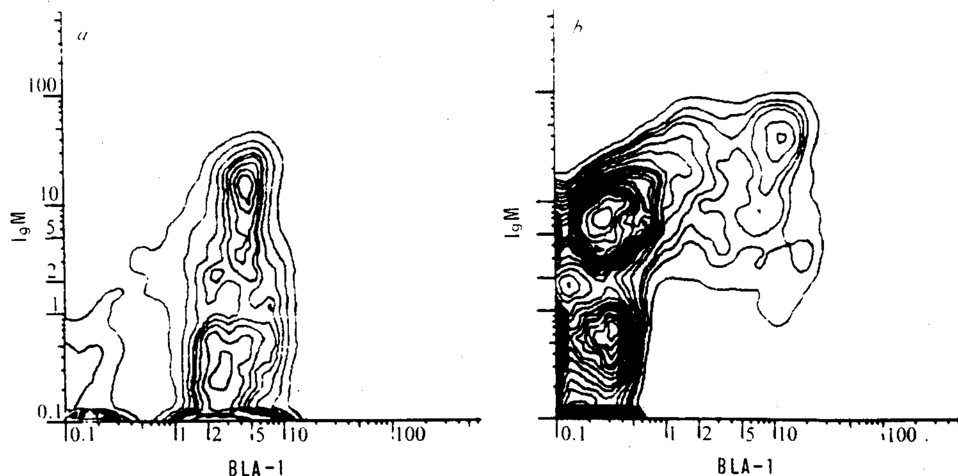

Fig. 2 All IgM^+ cells in the spleens of young animals are $BLA\text{-}1^+$ while only a portion of IgM^+ spleen cells in adults are $BLA\text{-}1^+$ (two-colour analyses). Spleen cells were from: CBA, 2 week old (*a*) and 2 month old (*b*) mice. See Table 1 for numerical data.
Methods: Spleen-cell suspensions were treated and stained as described in Fig. 1 legend. Green fluorescence: fluorescein-labelled rat monoclonal anti-BLA-1 antibody[6]. Red fluorescence: biotin-labelled rat monoclonal anti-IgM followed by Texas-red-labelled avidin.

and *xid* animals at 2 weeks of age. Also, as our two-colour studies showed, the correlated expression of IgM and IgD in the two types of mouse at this age is comparable. Surprisingly, we find in analyses of spleen cells from adult animals (Fig. 3) that BLA-1-positive cells have a similar IgM/IgD pattern in both normal and *xid* mice, and that the major difference in the IgM/IgD pattern between normal and *xid* B cells[4,5,13] is limited to the BLA-1-negative B cells. In CBA (normal) mice, $BLA\text{-}1^-$ cells are low-IgM/high-IgD (population I) whereas in CBA/N (***xid***) mice $BLA\text{-}1^-$ cells have high levels of both IgM and IgD (designated population II in 'normal' strains).

Note that although the IgM/IgD pattern of $BLA\text{-}1^+$ cells is qualitatively similar for both normal and *xid* splenic B cells, *xid* mice have more cells in population III (high-IgM/low-IgD) than normal mice. In general, cells in population III express low levels of Ly-1[5,13], previously considered to be a pan-T-cell antigen; *xid* mice have normal numbers of $Ly\text{-}1^+$ B cells[14], however, so the increase in population III comes from cells that lack Ly-1. Thus, *xid* B cells are not simply a subset of the B cells found in normal mice. That is, their $BLA\text{-}1^-$ cells are concentrated in population II rather than in population I (as in normals), and their $BLA\text{-}1^+$ cells include cells in population III for which we have not yet found a counterpart in normal adult mice.

Our working hypothesis is that BLA-1-positive B cells are the precursors of BLA-1-negative B cells. Thus splenic B cells from young animals consist mostly of immature BLA-1-positive B cells whereas spleen cells from adult animals include these cells (characterized by a very reproducible IgM/IgD pattern) and, in addition, have the more mature BLA-1-negative cells (characterized by a distinctive IgM/IgD pattern). We suggest that these immature BLA-1-positive B cells may be the easily tolerized cell type previously described in young animals[15-17]. If B-cell tolerization plays an important part in self-nonresponsiveness, then since B cells are continuously being derived from stem cells, there must be a considerable fraction of 'tolerizable' B cells even in the adult animal and perhaps these are the BLA-1-bearing B cells detectable in the adult spleen. Regardless of whether this hypothesis is correct, our data on B-cell sub-

Table 1 Expression of BLA-1 on B cells decreases from 100% in 2-week-old mice to <50% in adults

Strain	Age	$IgM^+/BLA\text{-}1^-$	$IgM^+/BLA\text{-}1^+$
CBA	2 weeks	<1	25
CBA/N	2 weeks	<1	20
CBA	2 months	20	15
CBA/N	2 months	15	10

Cells were stained and analysed as described in Fig. 2 legend. Values report per cent of total spleen cells in each population.

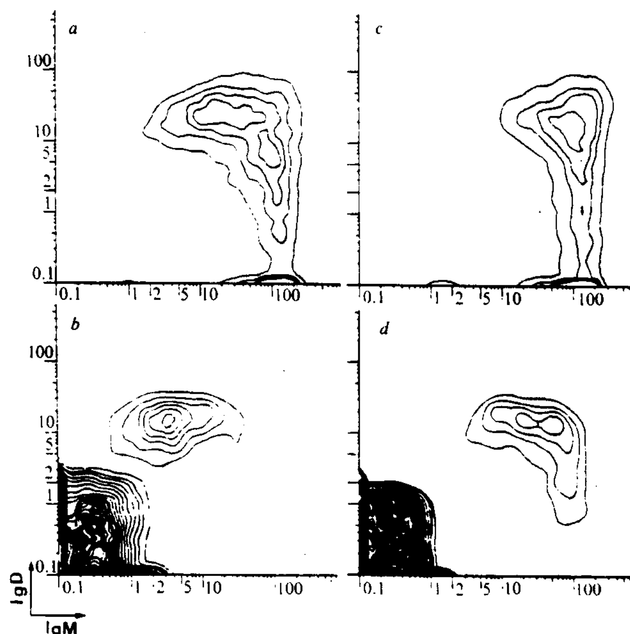

Fig. 3 IgM/IgD patterns for $BLA\text{-}1^+$ cells in adult spleen are similar for both normal and *xid* B cells, but very different for $BLA\text{-}1^-$ cells. IgM/IgD contour plots are shown for: *a*, CBA $BLA\text{-}1^+$ cells; *b*, CBA $BLA\text{-}1^-$ cells; *c*, CBA/N $BLA\text{-}1^+$ cells; *d*, CBA/N $BLA\text{-}1^-$ cells.
Methods: Three-colour immunofluorescence staining procedures were similar to the two-colour procedures described in Fig. 1 legend. Green fluorescence: fluoresceinated anti-IgM in the first incubation. Orange fluorescence: biotinated anti-IgD in the first incubation followed by PE-labelled avidin in the second incubation. Red fluorescence: APC-conjugated anti-BLA-1 antibody in the first incubation. The PE–avidin conjugate was prepared by cross-linking the two proteins with *N*-succinimidyl 3-(2-pyridyldithio)propionate[21] generously provided by Mr J. Kimura (B.D. Monoclonal Center, California). APC, a gift of Dr A. N. Glazer (University of California, Berkeley), was cross-linked to the 53–10.1 immunoglobulin[6] by first labelling the dye protein with succinimidyl 4-(*p*-maleimidophenyl)butyrate[22] and then reacting it with mildly reduced (using 20 mM dithiocrythritol) 10.1 antibody. Molecular-weight analysis by HPLC (TSK-250 column, Bio-Rad) showed that the conjugate consists primarily of one dye protein coupled to a single IgG. Stained cells were analysed on a dual-laser FACS modified to detect and process the extra fluorescence signal. Three-colour staining data is analysed by taking 'slices' of the histogram generated from one stain (for example $BLA\text{-}1^+$ cells) and examining the correlated expression of the two other markers (for example IgM and IgD) in this gated population. Such slices are displayed as two-colour contour plots.

populations have important implications for functional studies comparing B cells in normal and *xid* mice.

Many functional studies[18] examining B-cell development and responses rest on comparisons of results obtained using normal B cells (Lyb-3, 5, 7 positive and negative) with those obtained using *xid* B cells (Lyb-3, 5, 7 negative). This assumes that all B cells in *xid* mice are equivalent to the Lyb-3, 5, 7-negative subpopulation of B cells found in young or adult normal animals. We have now shown, however, that B cells from adult CBA/N (*xid*) mice are not equivalent to immature normal B cells and cannot simply be regarded as Lyb-3, 5, 7-negative cells from normal mice. Specifically, CBA/N mice have 'aberrant' $BLA\text{-}1^-$ cells (high-IgM/high-IgD) and further $BLA\text{-}1^-$ cells (high-IgM/low-IgD/$Ly\text{-}1^-$). Thus we suggest that great caution should be used in drawing conclusions from any study that substitutes CBA/N B cells for immature normal B cells or for normal $Lyb\text{-}5^-$ B cells.

We thank Dr P. Kincade for monoclonal rat anti-IgM, Mr H. Takahashi and Ms G. Putnam for technical assistance, Mr W. Moore for writing the VAX computer data handling programs and Professor L. A. Herzenberg for help and discussion. This work was supported in part by NIH grants CA-04681, HD-01287 and GM-17367. R.R.H. is a junior fellow of the American Cancer Society, California Division (grant J-22-82).

Received 18 July; accepted 26 August 1983.

1. Scher, I., Frantz, M. M. & Steinberg, A. D. *J. Immun.* **110**, 1396–1401 (1973).
2. Scher, I., Sharrow, S. O. & Paul, W. E. *J. exp. Med.* **144**, 507–518 (1977).
3. Mosier, D. E. *et al. Immun. Rev.* **37**, 89–104 (1977).
4. Hardy, R. R., Hayakawa, K., Haaijman, J. & Herzenberg, L. A. *Nature* **297**, 589–591 (1982).
5. Hardy, R. R., Hayakawa, K., Haaijman, J. & Herzenberg, L. A. in *Immunoglobulin D: Structure and Function* (eds Thorbecke, G. J. & Leslie, G. A.) 112–121 (New York Academy of Science, 1982).
6. Ledbetter, J. A. & Herzenberg, L. A. *Immun. Rev.* **47**, 63–90 (1979).
7. Huber, B., Gershon, R. K. & Cantor, H. *J. exp. Med.* **145**, 10–20 (1977).
8. Ahmed, A. *et al. J. exp. Med.* **145**, 101–110 (1977).
9. Subbarao, B. *et al. J. Immun.* **122**, 2279–2285 (1979).
10. Oi, V. T., Glazer, A. N. & Stryer, L. *J. Cell Biol.* **93**, 981–986 (1982).
11. Loken, M. R., Parks, D. R. & Herzenberg, L. A. *J. Histochem. Cytochem.* **25**, 899–907 (1977).
12. Parks, D. R., Hardy, R. R. & Herzenberg, L. A. *Immun. Today* **4**(5), 145–150 (1983).
13. Herzenberg, L. A. *et al. Immun. Rev.* **67**, 5–31 (1982).
14. Hayakawa, K., Hardy, R. R., Parks, D. R. & Herzenberg, L. A. *J. exp. Med.* **157**, 202–218 (1983).
15. Metcalf, E. S. & Klinman, N. R. *J. exp. Med.* **143**, 1327–1340 (1976).
16. Teal, J. M. & Klinman, N. R. *Nature* **288**, 385–387 (1980).
17. Nossal, G. J. V. & Pike, B. L. in *Immunology 80—Progress in Immunology IV* (eds Fougereau, M. & Dausset, J.) 136–152 (Academic, London, 1980).
18. Singer, A. *et al. Immun. Rev.* **64**, 137–160 (1982).
19. Kincade, P. W., Lee, G., Sun, L. & Watanabe, T. *J. immun. Meth.* **42**, 17–26 (1981).
20. Oi, V. T., Jones, P. P., Goding, J. W., Herzenberg, L. A. & Herzenberg, L. A. *Curr. Topics Microbiol. Immun.* **8**, 115–129 (1978).
21. Carlsson, J., Drevin, H. & Axen, R. *Biochem. J.* **173**, 723–737 (1978).
22. Lee, A. C. J., Powell, J. E., Tregear, G. W., Niall, H. D. & Stevens, V. C. *Molec. Immun.* **17**, 749–756 (1980).