immunoreactivity, a finding which agrees with earlier results showing that catecholamines co-exist with enkephalins in central and peripheral neurones[19]. It remains to be shown that the Leu-enkephalin-immunoreactive F9 cells also exhibit tyrosine hydroxylase immunofluorescence. In addition to cholinergic neurones, AChE activity has been demonstrated in cholinergic and adrenergic sympathetic neurones[20]. Substance P, on the other hand, has been localized mainly in sensory neurones[21], although recently it has also been found in some sympathetic neurones[22]. The absence of substance P-like immunoreactivity in F9 cells suggests, on the above hypothesis, that NGF does not favour sensory line differentiation. The NGF-dependent tyrosine hydroxylase immunoreactivity of the F9 cells demonstrate for the first time that NGF has the ability to induce adrenergic neuronal differentiation.

We thank Professor K. Unsicker for purified NGF, Dr A. Keller for tyrosine hydroxylase antibodies, Dr J. Polak for supplying antibodies for substance P, Leu- and Met-enkephalins and Dr D. Dahl for supplying antibodies for glial fibrillary acidic protein and neurofilament proteins.

Received 3 July; accepted 31 August 1983.

1. Graham, C. F. in *Concepts in Mammalian Embryogenesis* (ed. Sherman, M. I.) 315–394 (MIT Press, Cambridge, 1977).
2. Jacob, F. *Proc. R. Soc.* B**201,** 241–270 (1978).
3. Martin, G. R. *Science* **209,** 768–776 (1980).
4. Darmon, M., Buc-Caron, M.-H., Paulin, D. & Jacob, F. *EMBO J.* **1,** 901–906 (1982).
5. Paulin, D., Jakob, H., Jacob, F., Weber, K. & Osborn, M. *Differentiation* **22,** 90–99 (1982).
6. Adamson, E. D., Evans, M. J. & Magrane, G. G. *Eur. J. Biochem.* **79,** 607–615 (1977).
7. Pfeiffer, S. E. *et al. J. Cell Biol.* **88,** 57–66 (1981).
8. Kuff, E. L. & Fewell, J. W. *Devl Biol.* **77,** 103–115 (1980).
9. Levi-Montalcini, R. & Angeletti, P. U. *Physiol. Rev.* **48,** 534–690 (1968).
10. Nagatsu, T., Levitt, M. & Udenfriend, S. *Biochem. biophys. Res. Commun.* **14,** 543–549 (1964).
11. Wartiovaara, J., Leivo, I., Virtanen, I., Vaheri, A. & Graham, C. F. *Nature* **272,** 355–356 (1978).
12. Karnovsky, M. J. & Roots, L. *J. Histochem. Cytochem.* **12,** 219–221 (1964).
13. Liesi, P., Dahl, D. & Vaheri, A. *J. Cell Biol.* **96,** 920–924 (1983).
14. Levi-Montalcini, R. & Booker, B. *Proc. natn. Acad. Sci. U.S.A.* **42,** 384–391 (1960).
15. Schwartz, J. & Costa, E. *Brain Res.* **170,** 198–202 (1979).
16. Black, I. & Kessler, J. in *Neurosection and Brain Peptides* (eds Martin, J. B., Reichlin, S. & Bick, K. L.) 287–297 (Raven, New York, 1981).
17. Thoenen, H. & Barck, Y.-A. *Physiol. Rev.* **60,** 1284–1323 (1980).
18. Walicke, P. A. & Pattersson, P. H. *J. Neurosci.* **1,** 333–342 (1981).
19. Hökfelt, T. *et al. Proc. R. Soc.* B**210,** 63–77 (1980).
20. Eränkö, O. *A. Rev. Pharmac.* **7,** 203–222 (1967).
21. Hökfelt, T., Elfvin, L.-G., Schulzberg, M., Goldstein, M. & Nilsson, G. *Brain Res.* **132,** 29–41 (1977).
22. Kessler, J., Adler, J., Bohn, M. & Black, I. *Science* **214,** 335–336 (1981).
23. Dahl, D. & Bignami, A. *J. comp. Neurol.* **76,** 645–658 (1977).
24. Dahl, D. *Biochim. biophys. Acta* **668,** 299–306 (1981).
25. Berod, A., Hartman, B. K., Keller, A., Joh, T. H. & Pujol, J. F. *Brain Res.* **240,** 235–243 (1982).
26. Lamouroux, A. *Proc. natn. Acad. Sci. U.S.A.* **79,** 3881–3885 (1981).
27. Wharton, J. *et al. Invest. Cell Path.* **2,** 3–10 (1979).

# Glycyl glutamine, an inhibitory neuropeptide derived from β-endorphin

**D. C. Parish*‡, D. G. Smyth*§, J. R. Normanton†‡ & J. H. Wolstencroft†**

* National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
† University of Birmingham, Vincent Drive, Birmingham B15 2TJ, UK

**The primary mechanism of activation of intracellular prohormones seems to involve proteolytic cleavage at sequences of consecutive basic residues[1]. Thus, all the known biologically active peptides derived from the prohormone of corticotropin and β-endorphin appear to be excised initially by enzymes with this specificity. The C-terminal peptide, β-endorphin (1–31), is generated by cleavage at a lysyl arginine sequence and an additional cleavage can give rise to the related peptides, β-endorphin (1–27) and β-endorphin (1–26). These derivatives of β-endorphin are released by an endopeptidase that appears to catalyse cleavage on the carboxyl side of paired lysine residues, followed by the action of a carboxypeptidase B-like enzyme (Fig. 1). The β-endorphin fragments, β-endorphin (1–27) and β-endorphin (1–26), have been isolated from porcine[2–4] and bovine pituitary[5] but the C-terminal dipeptide, glycyl glutamine, has not been reported previously. Here we describe the isolation of glycyl glutamine from porcine pituitary and present evidence for its presence in sheep brain stem. When applied ionophoretically to brain stem neurones in the rat, the dipeptide exhibited an inhibitory action on cell firing.**

Glycyl glutamine was extracted from porcine pituitary and identified by ion-exchange chromatography (see Fig. 2 legend). The identity of the isolated dipeptide was confirmed by amino acid analysis, yielding glycine and glutamic acid in a ratio of 1.05 : 1.

Glycyl glutamine was shown to be present in porcine pituitary in substantial quantity (Table 1). When extracted from dissected regions of the pituitary, the dipeptide was found not only in the pars intermedia and posterior pituitary but also in the anterior pituitary. This was surprising because the 26- and 27-residue forms of β-endorphin occur only to a minor extent in the anterior pituitary and are concentrated in the pars intermedia[6,7]. It seems likely, therefore, that the glycyl glutamine in the anterior pituitary is derived from the β-endorphin prohormone or its 91-residue fragment, lipotropin, as well as from β-endorphin. In contrast, the glycyl glutamine present in the pars intermedia probably originates directly from β-endorphin, as little lipotropin or prohormone is present in that region.

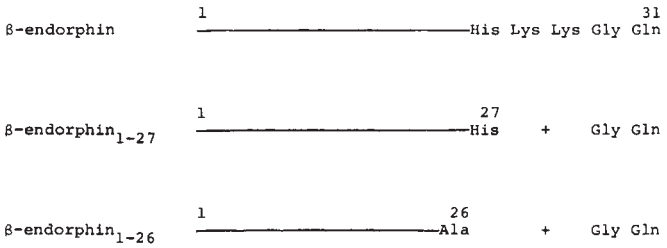


**Fig. 1** Diagrammatic representation of β-endorphin and its fragments in pituitary and brain.

Further experiments showed that glycyl glutamine occurs in the secretory vesicles of the pituitary. Granules obtained from 1.75 g of porcine pituitary[8] were lysed by sonication and the dipeptide was extracted and identified by chromatography and amino acid analysis. The results demonstrated that the dipeptide was present in the granule fraction (Table 1), where it seems to be elaborated together with the larger fragments of the ACTH–endorphin prohormone. We therefore conclude that the pituitary granules retain peptides of widely different molecular size, ranging from a dipeptide to a prohormone of 241 residues.

As β-endorphin and α-melanotropin (α-MSH) are known to occur in both $NH_2$-and *N*-acetylated states[9,10], experiments were carried out to determine whether glycyl glutamine might be elaborated also in an α,*N*-acetyl form. The procedure followed the same conditions as those used to identify the non-acetylated form of glycyl glutamine, synthetic α,*N*-acetylglycyl glutamine ($5 \times 10^4$ c.p.m., specific activity 20 Ci mmol$^{-1}$) being used as a marker peptide to guide the isolation of a putative acetylated form of glycyl glutamine. The tritium-labelled dipeptide was not retained during cation exchange chromatography and the excluded fraction obtained was shown by hydrolysis to be devoid of glutamic acid. Thus, the acetylated dipeptide does not exist to a significant extent in porcine pituitary. This indicates that during the processing of the ACTH–endorphin prohormone the enzymes that acetylate β-endorphin and α-MSH exhibit a

‡ Present addresses: National Institutes of Health, Bethesda, Maryland 20205, USA (D.C.P.); University Laboratory of Physiology, Parks Road, Oxford OX1 3PT, UK (J.R.N.).
§ To whom reprint requests should be addressed.

0028–0836/83/460267—04$01.00

**Table 1** Yields of glycyl glutamine obtained from porcine pituitary and ovine brain stem

| Source | Tissue weight (g) | Amount of Gly-Gln isolated | % Recovery | Content of Gly-Gln in initial tissue (nmol per g) |
|---|---|---|---|---|
| Whole pituitary | 5.1 | 98 nmol | 21 | 91.5 |
| Anterior pituitary | 6.15 | 51 nmol | 20.4 | 40.6 |
| Pars intermedia + posterior pituitary | 1.5 | 40 nmol | 24 | 111.1 |
| Pituitary secretory granules | 1.75 | 10 nmol | — | — |
| Ovine brain stem | 34.6 | 77 pmol | 19 | 11.7 |

The amounts of the dipeptide were measured on an LKB amino acid analyser Model 4400 and the recoveries were estimated from the quantity of $^3$H-labelled dipeptide that appeared in the chromatographed fraction as a percentage of the radioactivity added to the initial homogenate. Because $^3$H-glycyl glutamine would not co-centrifuge with the secretory granules, the marker in this case was added immediately before the gel filtration step and no overall yield was calculated.

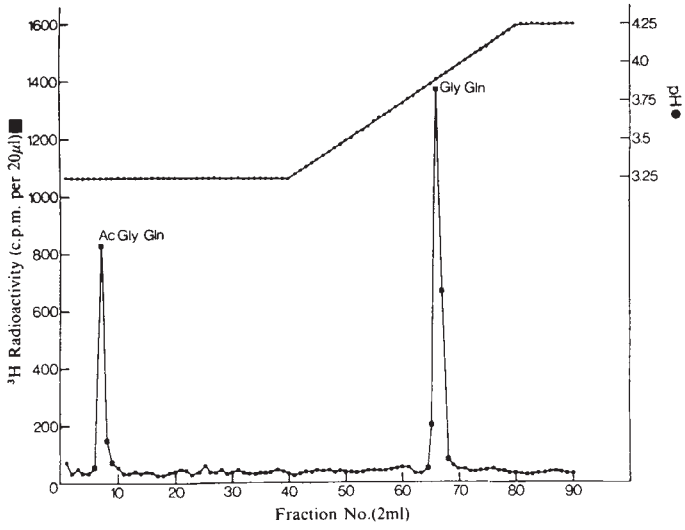


**Fig. 2** Ion exchange chromatography of glycyl glutamine extracted from porcine pituitary. To extract glycyl glutamine from the pituitary, the tissue (5.1 g) was homogenized in 50 ml of acid acetone (acetone, hydrochloric acid, $H_2O$, 40 : 1 : 6) in the presence of a trace amount of $^3$H-labelled glycyl glutamine (50,000 c.p.m., specific activity 20 Ci mmol$^{-1}$). Centrifugation was carried out at 25,000*g*, 4 °C, for 30 min and after concentration of the supernatant *in vacuo*, gel filtration was carried out on a column (70 × 2 cm) of Sephadex G-75 in 1 M acetic acid. Subsequent ion exchange chromatography of the $^3$H-containing fraction was on a column of IR-120 with volatile effluents. $^3$H-Glycyl glutamine and $^3$H-acetyl glycyl glutamine were added as marker peptides. The column (55 × 0.9 cm) of IR-120 was operated at 50 °C with a linear *p*H gradient from 0.2 M pyridine formate *p*H 3.25 to 0.2 M pyridine acetate *p*H 4.25 (mixer volume 100 ml) and flow rate 1 ml min$^{-1}$. The peptides in the eluted fractions (2 ml) were located by scintillation counting of aliquots. The material that eluted with the radiolabelled marker was applied to an amino acid analyser and the principal ninhydrin-reactive component was seen to chromatograph in the same position as synthetic glycyl glutamine, immediately after the elution position of valine.

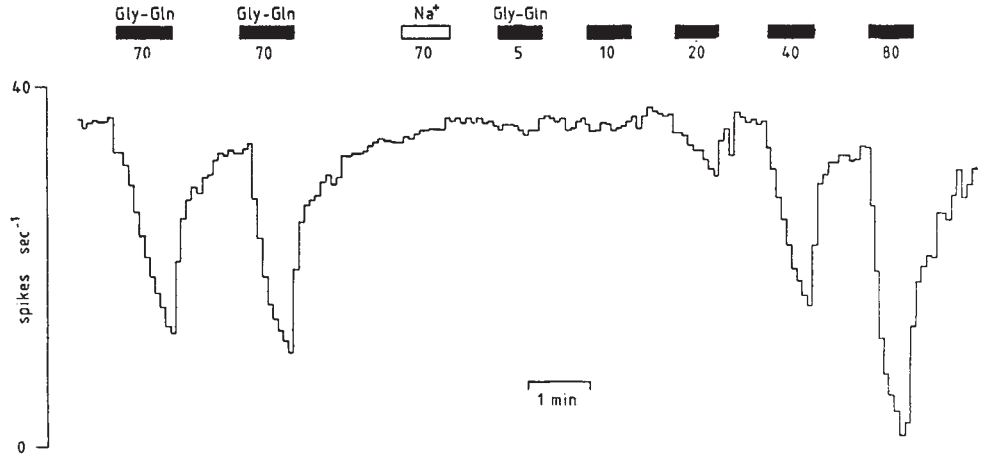


**Fig. 3** Inhibition of the firing rate of a neurone in the nucleus reticularis gigantocellularis of rat brain stem by microionophoretic application of glycyl glutamine. Note the slow time course of the inhibitions and their exponential decay. Responses to glycyl glutamine were dose dependent up to 80 nA. Numbers below the bars indicate ionophoretic current in nA and the ordinate represents mean firing rate of the neurone in spikes s$^{-1}$ in successive 5-s periods. Recordings were made through a barrel containing 4 M NaCl, *p*H 7. Glycyl L-glutamine was ejected as a cation from a barrel with a 50 or 100 mM solution adjusted to *p*H 3.5–4.5 with 0.5 M HCl. The experiments were performed on adult Sprague–Dawley rats (290–320 g) anaesthetized with urethane (1.5 g per kg intravenously). Single neurones in the brain stem were recorded extracellularly and drugs, including glycyl L-glutamine, were applied via five- or seven-barrelled glass micropipettes (tip diameter 5–8 μm)[13]. Drug ejections were routinely compared with current controls and were occasionally current compensated. Drug-induced excitations or inhibitions were considered significant when they exceeded 30% elevation or reduction in neuronal firing rate respectively. The locations of cells responsive to glycyl glutamine, or at least one cell per brain penetration, were marked by the ejection of Pontamine sky blue dye (100 μA min$^{-1}$) from one barrel of the micropipette followed by histological analysis of 50-μm frozen sections counterstained with Neutral red.

degree of specificity; they do not appear to act on the C-terminal dipeptide.

The formation of glycyl glutamine seems to involve proteolysis at the carboxyl side of the lysyl lysine residues at positions 28 and 29 of β-endorphin, as cleavage on the $NH_2$ side of these residues would release a tetrapeptide, Lys-Lys-Gly-Gln (Fig. 1). In the present experiments, glycyl glutamine was obtained from the pars intermedia in similar yield to the shortened forms of β-endorphin, which indicates that the four residues at the C-terminus of β-endorphin are unlikely to be excised as an intact tetrapeptide. Consequently, the synthetic peptide Lys-Lys-Gly-Glu, which has been reported to potentiate the melanotropic effect of α-MSH on anolis lizard skin[11], is unlikely to represent a natural fragment of β-endorphin.

It is well established that β-endorphin and its derivatives occur in brain as well as in pituitary, and it was therefore of

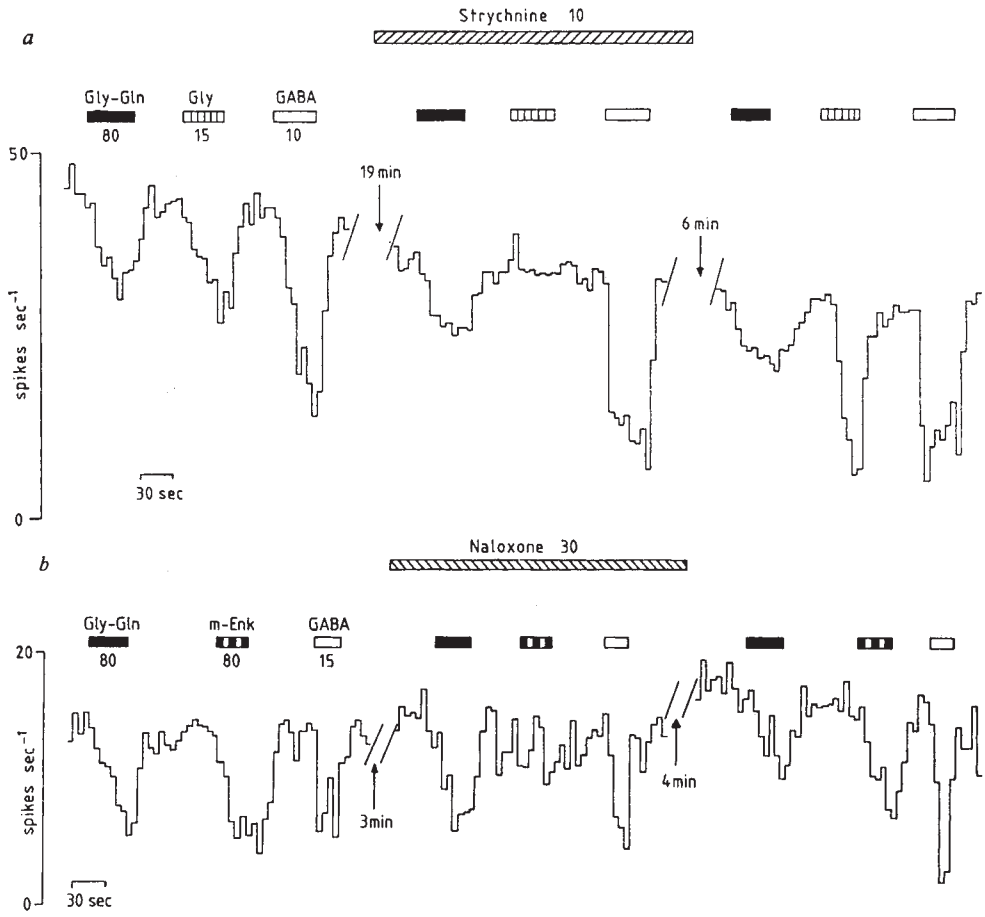


**Fig. 4** *a*, Absence of antagonism by strychnine of responses to glycyl glutamine (■) and GABA (□). Glycine responses were reversibly antagonized (▥). *b*, Absence of antagonism of glycyl glutamine responses by naloxone (▨). Inhibitory responses to [Met]enkephalin were seen in 8 out of 14 reticular neurones (57%); submaximal responses were reversibly antagonized. Glycine was ejected as a cation from a barrel containing a 10 mM solution at *p*H 4.5–5.0; strychnine as a cation from a solution of 10 mM strychnine HCl in 150 mM NaCl at *p*H 6; GABA as a cation from a 100 nM solution at *p*H 4.5–5.0, naloxone as an anion from a solution of 27 mM naloxone-HCl (Endo) at *p*H 4.5. In some experiments L-glutamine was ejected as a cation from a 100 mM solution at *p*H 7.5–8.0. Other details are given in Fig. 3 legend.

interest to demonstrate the presence of glycyl glutamine in the brain stem, a region where the truncated forms of β-endorphin have been shown to predominate[12]. Using the same procedure, the dipeptide was extracted from sheep brain stem (including medulla, pons and mid-brain) and identified by comparison of its chromatographic properties with those of synthetic glycyl glutamine. In addition, the shortened forms of β-endorphin were identified by chromatography and radioimmunoassay. The amounts of glycyl glutamine obtained (Table 1) corresponded to the total amounts of β-endorphin (1–27) and β-endorphin (1–26), consistent with its derivation from β-endorphin.

As glycyl glutamine was found to be present in brain and is generated by a mechanism involving cleavage at paired basic residues, the possibility was considered that the dipeptide might have a central function. Glycyl L-glutamine was therefore synthesized and its actions on brain stem neurones were investigated by microiontophoresis. We found that glycyl glutamine applied with currents of 0–100 nA inhibited the firing of 41 out of 109 neurones located within the nucleus reticularis gigantocellularis and nucleus reticularis pontis caudalis of the brain stem reticular formation. Effects were seen both on spontaneously active cells and on cells induced to fire by ejection of DL-homocysteic acid. Most inhibitions were slow in onset (~45–60 s) and the return to baseline firing rate after termination of the ejection current followed a similar time course (Fig. 3). However, a few responses (~10%) were more rapid, resembling the actions of γ-aminobutyric acid (GABA) and glycine (onset ~10–15 s). Both categories of response were dose dependent (see, for example, Fig. 3), typical minimum effective ejection currents being of the order of 40–60 nA applied for 30–35 s.

The actions of glycyl glutamine are not explained in terms of the actions of the constituent amino acids which could be liberated by proteolysis. Although inhibitory responses to glycine were seen on 19 of 21 cells, iontophoretic ejection of strychnine (5–15 nA), which abolished the responses to glycine, had no effect on submaximal responses to glycyl glutamine (Fig. 4*a*). Furthermore, in contrast to the inhibitory responses to glycyl glutamine applied at 40–60 nA, the only response observed with L-glutamine at >80 nA was a weak excitation (~25–30% increase in firing rate) in 3 out of 15 cells.

In view of the relationship between glycyl glutamine and β-endorphin, the possibility was considered that the actions of the dipeptide were related to analgesia. It was observed, however, that glycyl glutamine inhibited a similar proportion of neurones (30–40%) responding to either noxious or non-noxious peripheral stimulation (strong mechanical pinch to skin/hindpaws or light touch/hair displacement, respectively). In addition, the inhibitory actions of glycyl glutamine on cells that responded to low-threshold proprioceptive inputs in the dorsal column nuclei indicated that its actions are not specifically related to pain. Note also that the actions of the dipeptide are not explained in terms of 'opioid-like' effects, as ionophoretic ejection of naloxone (20–40 nA), which abolished the responses to [Met]enkephalin, had no effect on submaximal responses to glycyl glutamine (Fig. 4*b*).

The results show that glycyl glutamine occurs as a natural peptide which can influence the firing of neurones in the central nervous system. Its actions are not mediated by receptors sensitive to strychnine or naloxone. Therefore, the intracellular proteolysis of β-endorphin which occurs in pituitary and brain does not only lead to attenuation of opioid properties; it appears to generate a new biological activity.

We thank Dr S. D. Lovell for assistance in providing samples of sheep brain. J.R.N. was supported by a grant from the MRC to J. H. Wolstencroft. We deeply regret the death of J. H. Wolstencroft on 2 May 1983.

Received 19 May; accepted 2 September 1983.

1. Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J. & Tager, H. S. *Ann. N. Y. Acad. Sci.* **343**, 1–16 (1980).
2. Bradbury, A. F., Smyth, D. G. & Snell, C. R. *Ciba Fdn Symp.* **41**, (new ser.) 61–74 (1976).
3. Smyth, D. G., Snell, C. R. & Massey, D. E. *Biochem. J.* **175**, 261–270 (1978).
4. Smyth, D. G., Smith, C. C. & Zakarian, S. in *Advances in Endogenous and Exogenous Opioids* (ed. Takagi, H.) 145–148 (Kodansha/Elsevier, Tokyo–Amsterdam, 1981).

5. Massey, D. E. & Smyth, D. G. *Biochem. Soc. Trans.* **8**, 751–752 (1980).
6. Smyth, D. G. & Zakarian, S. *Nature* **288**, 613–615 (1980).
7. Smyth, D. G., Zakarian, S., Deakin, J. F. W. & Massey, D. E. *Ciba Fdn Symp.* **81**, 79–96 (1981).
8. Jacobs, L. S., McKeel, D. W., Jarett, I. & Daughaday, W. H. *Endocrinology* **92**, 477–486 (1973).
9. Smyth, D. G., Massey, D. E., Zakarian, S. & Finnie, M. D. A. *Nature* **279**, 252–254 (1979).
10. Harris, J. J. & Lerner, A. B. *Nature* **179**, 1346–1347 (1957).
11. Carter, S. J., Shuster, S. & Morley, J. *Nature* **279**, 74–75 (1979).
12. Zakarian, S. & Smyth, D. G. *Nature* **296**, 250–252 (1982).
13. Normanton, J. R., West, D. C. & Wolstencroft, J. R. *Br. J. Pharmac.* **77**, 477–485 (1982).

# 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).

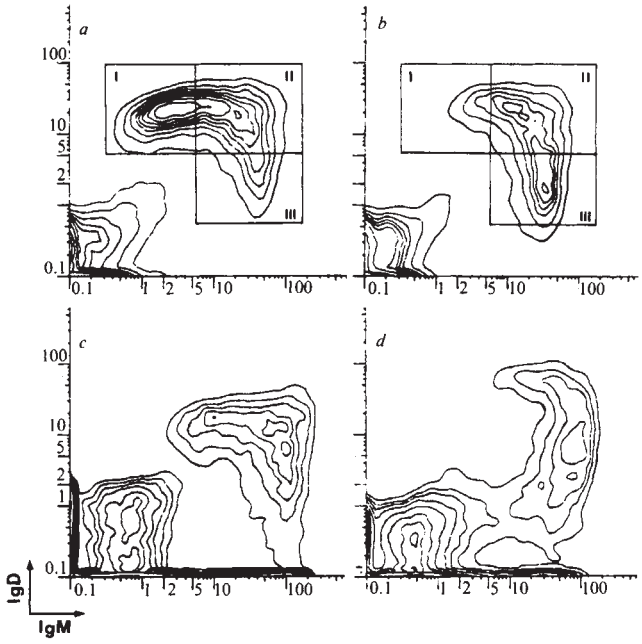


**Fig. 1** CBA/N mice lack a population of B cells characterized by high levels of IgD and intermediate to low levels of IgM (two-colour analyses). IgM/IgD-stained spleen cells are shown from: *a*, CBA, 3 months old; *b*, CBA/N, 3 months old; *c*, CBA, 2 weeks old; and *d*, CBA/N, 2 weeks old. The boundaries 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.

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

0028–0836/83/460270—03$01.00