8. K. DeGeest, M. Thiery, G. Piron-Possuyt, R. Driessche, *J. Perinat. Med.* **13**, 3 (1985).
9. H. H. Zingg and D. L. Lefebvre, *Mol. Brain Res.* **4**, 1 (1988).
10. To determine OT mRNA abundance in uteri and hypothalami of 21-day-pregnant rats, we densitometrically scanned autoradiograms obtained from nine independent blots as described [H. H. Zingg, G. Almazan, D. L. Lefebvre, *J. Biol. Chem.* **261**, 12956 (1986)]. On each autoradiogram, band intensities were normalized with respect to the amount of RNA loaded per lane (5 to 50 μg), and the ratio of uterine versus hypothalamic OT mRNA abundance was determined. From these measurements, the uterus of a 21-day-pregnant rat contained 2.3 ± 0.6 times more OT mRNA per microgram of total RNA than the hypothalamus of a rat at the same stage of pregnancy (mean ± SE; $n = 9$). The amount of total RNA extracted from one uterus or one hypothalamus was also determined. One whole uterus of a 21-day-pregnant rat was found to have 30 times more total RNA than a hypothalamus (6502 ± 1659 μg in the uterus versus 219.6 ± 24.1 μg in the hypothalamus; means ± SE; $n = 9$). Taken together, these results indicate that at day 21 of pregnancy, the whole rat uterus contains about 70 times more OT mRNA than the hypothalamus. We have also recently identified the presence of OT mRNA in rat placenta (*15*). However, placental OT mRNA levels are over 10 times lower than hypothalamic OT mRNA levels and over 20 times lower than uterine OT mRNA levels (*15, 21*).
11. R. Ivell and D. Richter, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2006 (1984).
12. The radioimmunoassay was performed as described (*15*) with a rabbit antibody to OT (Cal Biochem), $^{125}$I-labeled OT, and synthetic standards (Peninsula Laboratories, Belmont, CA). The antibody was used at a final dilution of 1:7500. Specific binding of tracer in the absence of added hormone ranged from 20 to 25%. Assay sensitivity was 2 pg per tube. The antiserum used showed no measurable cross-reactivity with arginine vasopressin and had a greater than 100-fold lower affinity for vasotocin or lysine vasopressin [R. C. Gorewit, *Proc. Soc. Exp. Biol. Med.* **160**, 80 (1979)].
13. Molecular sizing was performed as described. HPLC fractions eluting at 44 to 48 min (Fig. 3, second peak) were pooled, frozen, dried by evaporation, and resuspended in 0.1% trifluoroacetic acid (TFA). Portions were either analyzed directly or subjected to three cycles of freezing and thawing. Samples were loaded on two I125 gel filtration columns (Waters) connected in series and eluted with 40% acetonitrile in $H_2O$ that contained 0.1% TFA at a flow rate of 1 ml/min. Fractions were collected, lyophilized, and assayed for ir-OT by RIA.
14. L. Shukovski, J. K. Findlay, A. I. Smith, *J. Endocrinol.* **128**, 305 (1991).
15. D. L. Lefebvre, A. Giaid, H. H. Zingg, *Endocrinology* **130**, 1185 (1992).
16. M. Akerlund *et al.*, *Brit. J. Obstet. Gynaecol.* **94**, 1040 (1987); L. Wilson, Jr., M. T. Parson, G. Flouret, *Am. J. Obstet. Gynecol.* **163**, 1875 (1990).
17. V. J. Ayad, S. E. F. Guldenaar, D. C. Whates, *J. Endocrinol.* **128**, 187 (1991); A. R. Fuchs, F. Fuchs, P. Husslein, M. S. Soloff, M. J. Fernström, *Science* **215**, 1396 (1982); J. M. Wallace *et al.*, *J. Endocrinol.* **128**, 253 (1991).
18. F. F. Ciarochi, A. G. Robinson, J. G. Verbalis, S. M. Seif, E. A. Zimmerman, *Peptides (New York)* **6**, 903 (1985).
19. M. S. Soloff, M. Alexandrova, M. J. Fernstrom, *Science* **204**, 1313 (1979).
20. I. W. Barash, W. Cromlish, B. I. Posner, *Endocrinology* **122**, 1151 (1988); S. Collins *et al.*, *J. Biol. Chem.* **265**, 19330 (1990); H. de Thé, M. Vivanco-Ruiz, P. Tiollais, H. Stunnenberg, A. Dejean, *Nature* **343**, 177 (1990).
21. D. L. Lefebvre, A. Giaid, H. Bennett, R. Larivière, H. H. Zingg, unpublished results.
22. M. Goedert, *EMBO J.* **6**, 3627 (1987).
23. S. M. Hsu, L. Raine, H. Fanger, *J. Histochem. Cytochem.* **29**, 577 (1981).
24. R. C. Graham and M. J. Karnovsky, *ibid.* **14**, 291 (1966).
25. D. L. Lefebvre and H. H. Zingg, *Mol. Endocrinol.* **5**, 645 (1991).
26. H. H. Zingg, R. H. Goodman, J. F. Habener, *Endocrinology* **115**, 90 (1984).
27. Reversed-phase HPLC was performed with a model 510 liquid chromatography system and a 3.9 mm by 30 cm μBondapak C18 column (Waters). Samples were loaded onto the column in 0.1% TFA and eluted at a flow rate of 1.5 ml/min over 60 min with a linear gradient of acetonitrile and TFA. Fractions were collected for 1 min, lyophilized, and reconstituted in 1 ml of a solution that contained 0.01 N acetic acid and 0.1% bovine serum albumin. Portions (100 μl) were assayed for OT peptide content by RIA (*12*).
28. For in situ hybridization, sections were treated with proteinase K, fixed in 4% paraformaldehyde, washed with 2× sodium citrate buffer (SSC) (1× SSC contains 0.15 M NaCl and 0.015 M sodium citrate) and incubated with prehybridization buffer [50% formamide, SSC (5×), 50 mM sodium phosphate buffer (pH 7.0), salmon sperm DNA (250 μg/ml), 0.1% Ficoll (Pharmacia), 0.1% polyvinyl pyrrolidone, 0.1% bovine serum albumin, heparin (200 μg/ml), and 0.1% SDS] for 2 hours at room temperature (*22*). Sections were then incubated with the solution above containing $1 \times 10^6$ cpm per section of $^{35}$S-labeled probe for 16 hours at 42°C. Washes were performed in 2× SSC, 1× SSC, and 0.5× SSC for 1 hour each at room temperature and in 0.5× SSC at 42°C for 30 min. Sections were washed in increasing concentrations of ethanol (70%, 90%, and 100%, for 10 min each), air-dried, dipped in Ilford K-5 emulsion, and exposed for 1 to 2 weeks at 4°C. Sections were counterstained in hematoxylin.
29. For immunocytochemistry, paraffin was removed from sections. After permeabilization with Triton X-100 (0.2%), sections were incubated overnight with rabbit antiserum to OT (Peninsula Laboratories.) at a dilution of 1/1000 at 4°C. The sections were immunostained by the avidin-biotin complex method (*23*) with biotinylated goat antibody–rabbit immunoglobulin G and an avidin-biotin complex (Vector Labs). The peroxidase reaction was revealed by the diaminobenzidine method (*24*). Sections were hematoxylin counterstained. As controls of specificity, some sections were incubated with nonimmune serum instead of the primary antiserum or one of the other staining procedures was omitted.
30. R. Chibbar, F. Miller, B. F. Mitchell, *Proceedings of the 39th Annual Meeting of the Society for Gynecologic Investigation*, San Antonio, TX, 18 March 1992, p. 251.
31. We thank J. Neculcea and C. Charbonneau for their expert assistance. Supported by grants from the Medical Research Council of Canada. D.L.L. is supported by a studentship from the Medical Research Council of Canada. A.G. is a recipient of a scholarship from the Heart and Stroke Foundation of Canada.

13 January 1992; accepted 17 April 1992

# Early Stages of Motor Neuron Differentiation Revealed by Expression of Homeobox Gene *Islet-1*

Johan Ericson, Stefan Thor, Thomas Edlund, Thomas M. Jessell,* Toshiya Yamada

**Motor neurons in the embryonic chick spinal cord express a homeobox gene, *Islet-1*, soon after their final mitotic division and before the appearance of other differentiated motor neuron properties. The expression of Islet-1 by neural cells is regulated by inductive signals from the floor plate and notochord. These results establish Islet-1 as the earliest marker of developing motor neurons. The molecular nature of the Islet-1 protein suggests that it may be involved in the establishment of motor neuron fate.**

During embryonic development the vertebrate nervous system generates a diverse array of neuronal cell types, which are characterized by their position of origin, axonal projections, and synaptic connections. One of the neuronal types for which the processes of axonal pathfinding and synapse formation have been documented in most detail is the spinal motor neuron (*1*). In contrast, the events that control the generation of motor neurons remain largely obscure. In chick embryos, spinal motor neurons derive from progenitor cells in the neural tube that give rise also to other neurons and to glial cells (*2*). The commitment of neural progenitors to a motor neuron fate appears to be regulated, in part, by signals that derive from axial mesodermal cells of the notochord and floor plate cells at the ventral midline of the neural tube (*3–5*). Insight into the molecular mechanisms involved in the generation of motor neurons requires the identification of genes that are expressed at the initial stages of motor neuron differentiation. Here we report that embryonic chick motor neurons express the homeobox gene *Islet-1* (6), a member of the subfamily of homeobox genes (*7*) that contain cysteine-rich *Lin-11*, *Isl-1 Mec-3* (LIM) domains (6, *8*, 9). Other members of this family include *Lin-11* and *Mec-3*, which have been shown to regulate cell fate in *Caenorhabditis elegans* (*8*, 9).

Islet-1 was originally identified as a protein that binds to enhancer elements in the rat insulin gene (6). In the adult rat, Islet-1

J. Ericson, S. Thor, T. Edlund, Department of Microbiology, Umeå University, Umeå S-90187, Sweden.
T. M. Jessell and T. Yamada, Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, Center for Neurobiology and Behavior, Hammer Health Sciences Center, Columbia University, New York, NY 10032.

*To whom correspondence should be addressed.

is expressed in pancreatic islet cells and in a subset of neurons, including motor neurons (*10*). To determine the earliest expression of Islet-1 during neural development, we localized the protein by immunocytochemistry in chick embryos (*11*). Islet-1 immunoreactivity is first detected in the nuclei of cells in the ventral region of the spinal cord, lateral to the floor plate (Fig. 1A). The onset of Islet-1 expression in cells in brachial regions of the spinal cord occurs at Hamburger-Hamilton (*12*) stage 15 and in lumbar regions at stage 17. Between stages 15 and 23 to 24, the number of Islet-1–positive cells in the ventral spinal cord increases markedly (Fig. 1, B and C, and Table 1) (*13*), and there is no substantial addition to the number of Islet-1–positive cells at stages 28 to 29 (Fig. 1D and Table 1). The location and time course of appearance of Islet-1–positive cells in the ventral spinal cord are similar to those of motor neurons, as defined by [$^3$H]thymidine birthdating studies (*14*). The expression of Islet-1 in ventral spinal cord neurons is maintained at later developmental stages (Fig. 1D) and in the adult (*10*).

To establish the identity of the ventral spinal cord cells that express Islet-1, we labeled motor neurons by retrograde transport after injections of horseradish peroxidase (HRP) into the ventral root of stage 24 chick embryos (*15*). More than 80% of Islet-1–positive cells in the ventral spinal cord had accumulated HRP in their cytoplasm (Fig. 2, A and B). Islet-1–positive cells that did not contain HRP were located predominantly in the medial region of the spinal cord (Fig. 2, A and B). These medial Islet-1–positive cells are likely to represent motor neurons that have only recently completed their final mitosis (*14, 16*). Thus, the absence of HRP in medial Islet-1–positive cells probably reflects the fact that they have not yet extended axons into the ventral root. These results suggest that Islet-1 is expressed by most and probably by all motor neurons in the spinal cord.

At thoracic and lumbar levels of the spinal cord, the single ventrolateral column of Islet-1–positive neurons begins to divide into two distinct cell groups, one of which remains in a ventrolateral position (Fig. 1C). The second group of Islet-1–positive cells is found at progressively more medial locations from stages 24 to 29 (Fig. 1D). The position of this medial group of Islet-1–positive cells suggests that they correspond to preganglionic motor neurons of the sympathetic nervous system (*16*). Islet-1 expression in these cells also persists at later developmental stages. These observations suggest that both somatic and visceral motor neurons express Islet-1.

In addition to the early expression of Islet-1 in ventral neurons, a few (one to ten cells per section) Islet-1–positive cells are present in the dorsal spinal cord beginning around stage 24 (Fig. 1C). These cells do not express other markers of embryonic spinal motor neurons, and their position does not correspond with that of any previously identified group of motor neurons. From stage 24 the number of Islet-1–positive cells in the most dorsal region of the spinal cord decreases, and there is a corresponding increase in the number of cells in the lateral regions of the dorsal spinal cord (Fig. 1D). Cells in the dorsal region of the chick spinal cord migrate ventrolaterally soon after their final mitotic division (*17*).

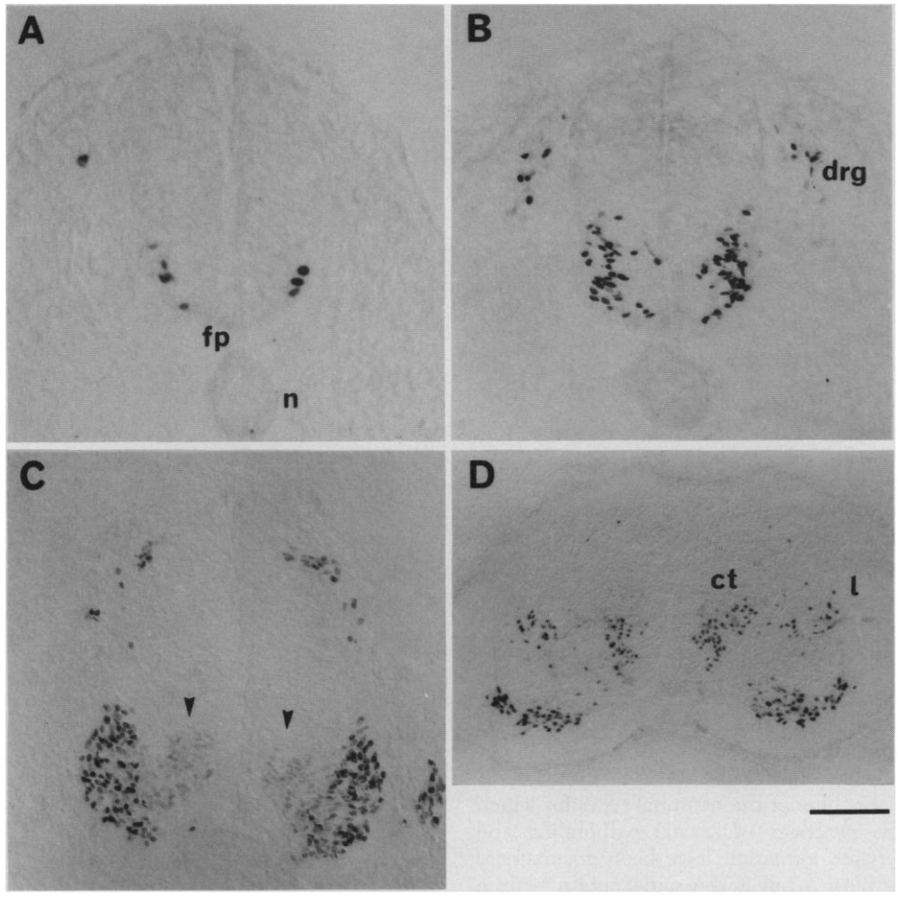


**Fig. 1.** Expression of Islet-1 during embryonic chick spinal cord development. (**A**) Cross section of the upper thoracic spinal cord of a stage 15 to 16 embryo. Islet-1 protein was detected with antibody to Islet-1 and HRP-conjugated secondary antibody. Islet-1 is expressed in a small number of cells in the ventral part of the spinal cord lateral to the floor plate (fp) and notochord (n). (**B**) By stages 17 to 18, there is a marked increase in the number of Islet-1–positive cells in the ventral spinal cord. Note that cells close to the midline of the spinal cord express Islet-1. Islet-1 is also expressed in cells of the dorsal root ganglion (drg). (**C**) Section through thoracic spinal cord at stage 24, showing Islet-1 expression in cells in the ventrolateral spinal cord. The medial-most groups of cells (arrowheads) appear to exhibit lower amounts of Islet-1 immunoreactivity at this stage. A small number of cells in the dorsal region of the spinal cord also express Islet-1. (**D**) Section through thoracic spinal cord at stage 29, showing Islet-1 expression in the cells in the lateral motor column and in presumptive visceral motor neurons that are beginning to form the Column of Terni (ct) (*16*). A lateral group (L) of Islet-1–positive cells is also present. Scale bar: (A) 60 μm; (B and C) 70 μm; (D) 90 μm.

**Table 1.** Developmental increase in Islet-1–positive cells in the ventral spinal cord. The number of Islet-1–positive cells was determined as described (*13*) in sections of the brachial spinal cord. Values given are mean ± SEM with the number of sections counted given in parentheses. Sections were obtained from two to four embryos at each developmental stage. We did not determine the total number of Islet-1–positive cells in specific motor columns, which would have required performing counts at later stages when individual motor columns were resolvable.

| Stage | Number of Islet-1–positive cells |
|---|---|
| 15–16 | 5 ± 1 (21) |
| 17–19 | 59 ± 2 (5) |
| 23–24 | 267 ± 7 (5) |
| 28–29 | 284 ± 28 (5) |

Thus, it is likely that the initial dorsal group of Islet-1–positive cells migrates ventrally and settles at an intermediate position along the dorsoventral axis of the spinal cord.

If Islet-1 has a function at initial stages of motor neuron differentiation, its expression would be expected to precede that of other defined properties of embryonic motor neurons. We therefore compared the onset of expression of Islet-1 with that of SC1, a glycoprotein of the immunoglobulin superfamily that is the earliest known surface marker of motor neuron differentiation in chick spinal cord (*18*). At around stage 15 the majority of Islet-1–positive motor neurons had not yet begun to express detectable amounts of SC1 (Fig. 2C, 1). By stages 16 to 17, approximately 70% of the Islet-1–positive cells also expressed SC1 (Fig. 2C, 2), and, by stages 25 to 26, virtually all ventral Islet-1–positive cells expressed SC1. This static analysis provides evidence that the expression of Islet-1 by embryonic motor neurons precedes that of SC1.

Studies in developing mammalian cerebral cortex have provided evidence that the laminar fate and axonal projections of cortical neurons are determined before the final division of precursor cells in the ventricular zone (*19*). However, the relation between terminal mitosis and determination of the identity of most neuronal types in the vertebrate central nervous system, including motor neurons, remains unclear. The presence of Islet-1–positive cells in the germinal region (*20*) of the ventral spinal cord at stages 15 to 20 (Figs. 1B and 2D) raised the possibility that Islet-1 is expressed by motor neuron precursors before their final cell division. To test this possibility, we exposed chick embryos at stages 15 to 16 to bromodeoxyuridine (BrdU) (100 μM) to label cells in the S phase of the cell cycle (*21*). We determined the proportion of Islet-1–positive cells that incorporated BrdU by immunocytochemistry at different times after the onset of BrdU application. At times up to 4 hours, less than 1% of Islet-1–positive cells had incorporated BrdU (Table 2). From 8 hours onward, the proportion of Islet-1 cells that coexpressed BrdU increased markedly (Table 2). Because the cell cycle time of neuroepithelial cells in the chick spinal cord at this stage is about 8 hours, with the time from S phase to M phase approximately 6 hours (*22*), these and other experiments (*23*) suggest that Islet-1 is expressed after the final division of motor neuron progenitors.

Studies on the expression of SC1 by chick spinal cord cells in vivo have provided evidence that the differentiation of motor neurons is dependent on inductive signals from the notochord and floor plate (*4, 5*). The identification of Islet-1 as an early marker of motor neuron differentiation led us to examine whether manipulations that affect the differentiation of motor neurons are associated with changes in the pattern of expression of Islet-1. The location of cells expressing Islet-1 was therefore examined in the spinal cord of chick embryos that had received grafts of the notochord or

**Table 2.** Percentage of Islet-1–positive–labeled ventral spinal cord cells that coexpress BrdU. The percentage of Islet-1–positive cells in the ventral spinal cord that coexpress BrdU immunoreactivity was determined as described (*21, 23*). Numbers in parentheses refer to the total number of ventral Islet-1–positive cells counted in sections derived from up to four embryos. ND, not determined.

| Stage | Time after BrdU application (hours) | | | | |
|---|---|---|---|---|---|
| | 1 | 4 | 8 | 12 | 24 |
| 15–16 | ND | 0.4 (247) | 20.5 (946) | 17.3 (178) | 55.9 (924) |
| 17–18 | 0.5 (347) | 1.1 (202) | 9.2 (142) | 18.0 (60) | 54.0 (175) |

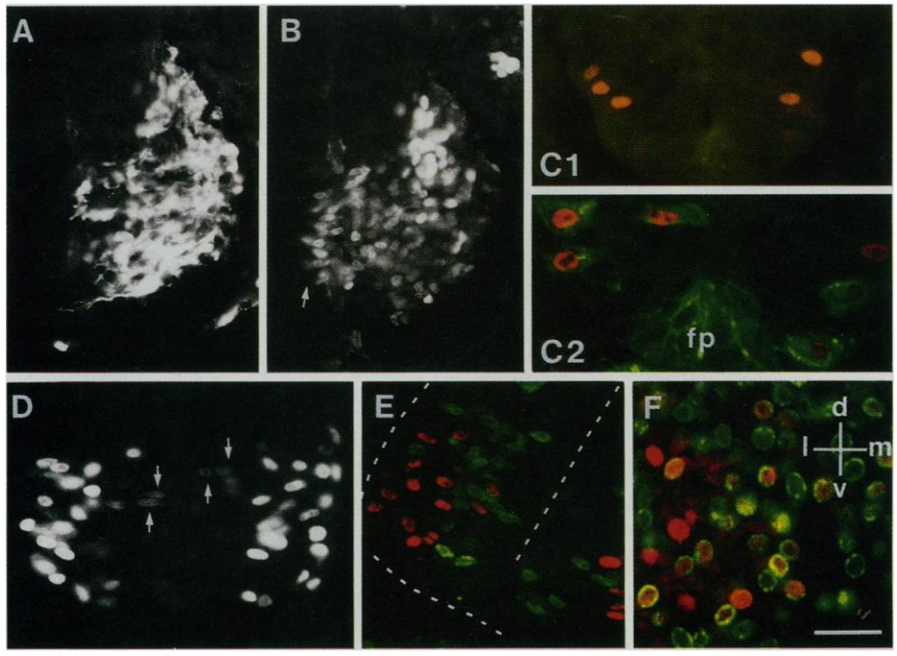


**Fig. 2.** Islet-1 expression by postmitotic motor neurons in chick spinal cord. (**A**) Immunofluorescent micrograph showing the expression of HRP in cells of the lateral motor column after injection of HRP into the ventral roots of stage 24 embryos. (**B**) The same section shown in (A) was double-labeled with rabbit antibody to Islet-1 and Texas Red–conjugated goat antibody to rabbit immunoglobulin. The majority of Islet-1–positive cells express HRP immunoreactivity in their cytoplasm. This was confirmed by confocal microscopy. The most medial Islet-1–positive cells (arrow) do not contain HRP. (**C**) Dual-color immunofluorescent images of the notochord and ventral spinal cord of stage 15 and 16 embryo. (C1) Section showing ventral spinal cord of a stage 15 embryo double-labeled with antibodies to Islet-1 and SC1. Ventral Islet-1–positive cells do not express SC1. Weak SC1 labeling is detected in the floor plate, and strong SC1 labeling is present in the notochord. (C2) Section of ventral spinal cord of a stage 15 to 16 embryo showing coexpression of Islet-1 (red) and SC1 (green) by motor neurons. Cells of the floor plate (fp) express SC1 but not Islet-1. The color micrograph was obtained by computer enhancement of a 2-μm confocal image of a labeled cryostat section. (**D**) Immunofluorescence micrograph showing the ventral half of the brachial spinal cord of a stage 16 to 17 embryo. Islet-1 was visualized with antibody to Islet-1 and Texas Red–conjugated goat antibody to rabbit immunoglobulin. Many cells near to the midline of the spinal cord express the Islet-1 protein (arrows), although at lower concentrations than lateral cells. (**E**) Confocal image of a section of stage 15 to 16 brachial spinal cord, outlined by dashes, fixed 4 hours after BrdU application. BrdU (green) and Islet-1 (red) are present in the nuclei of distinct cells. After a 4-hour incubation period, essentially no cells express both Islet-1 and BrdU. (**F**) Confocal image of the ventrolateral region of a section of brachial spinal cord from a stage 15 to 16 embryo exposed to BrdU and incubated for 24 hours. Many Islet-1–positive cells have incorporated BrdU as assessed by the yellow-orange nuclear label. Orientation of the section is shown (d, dorsal; v, ventral; m, medial; l, lateral). Scale bar: (A and B) 40 μm; (C1) 40 μm; (C2) 30 μm; (D and E) 35 μm; (F) 50 μm.

floor plate (*24*). The spinal cord of embryos that had received such grafts contained additional, apparently ectopic Islet-1–positive cells (Fig. 3, A and C) within 24 hours of graft implantation. The induced Islet-1–positive cells also expressed the SC1 glycoprotein and projected axons out of the spinal cord (Fig. 3, B and D) consistent with their identity as motor neurons. Thus, the presence of large numbers of Islet-1–positive cells in the dorsal spinal cord after

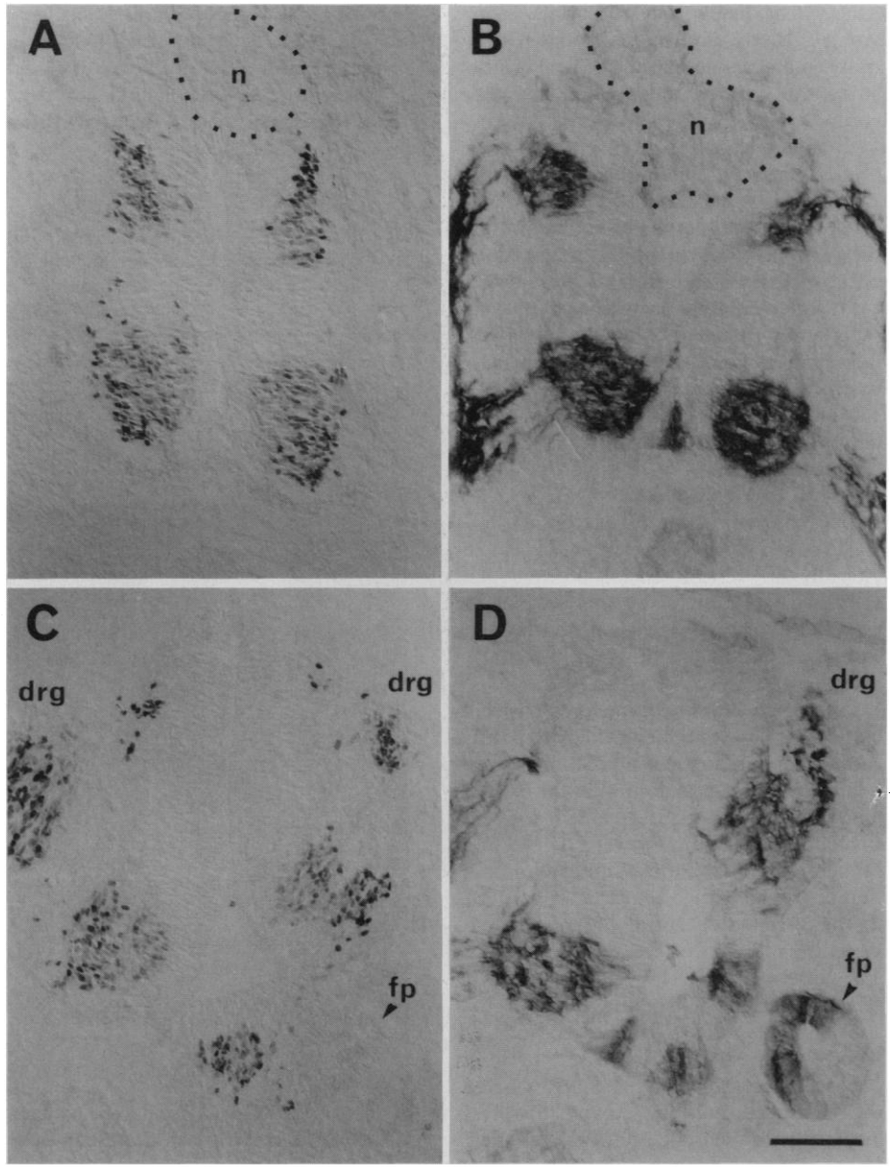


**Fig. 3.** Induction of ectopic Islet-1 expression in chick spinal cord cells by floor plate and notochord grafts. Section of spinal cord from an embryo in which a piece of stage 10 chick notochord (n) was grafted at the dorsal midline of the neural tube of stage 9 to 10 host embryos, which were incubated for a further 48 hours. The 10-μm cryostat sections were stained alternatively with antibodies to Islet-1 (A) and SC1 (B) and HRP-conjugated antibodies to rabbit or mouse immunoglobulins. (**A**) A large number of Islet-1–positive cells are present in two columns in the dorsal spinal cord. (**B**) A nearby section from the same embryo showing that SC1 is also induced in cells that form two dorsal columns. (**C** and **D**) Sections of spinal cord from embryos in which a small segment of stage 17 to 18 chick floor plate (fp) was grafted lateral to the neural tube of stage 9 to 10 host embryos, which were incubated for 48 hours. Serial sections were stained alternately with antibodies to Islet-1 (C) or SC1 (D). In (D), expression of SC1 shows that an ectopic floor plate is induced in the spinal cord adjacent to the position of the floor plate graft (arrowhead). Additional SC1-labeled motor neurons are induced on the operated side. (C) shows that Islet-1 is expressed in dorsal cells but not in the induced floor plate. A small number of cells in the dorsal spinal cord also express Islet-1 (C) but not SC1 (D). Dorsal root ganglion (drg) neurons also express both Islet-1 and SC1. Scale bar: (A to D) 70 μm.

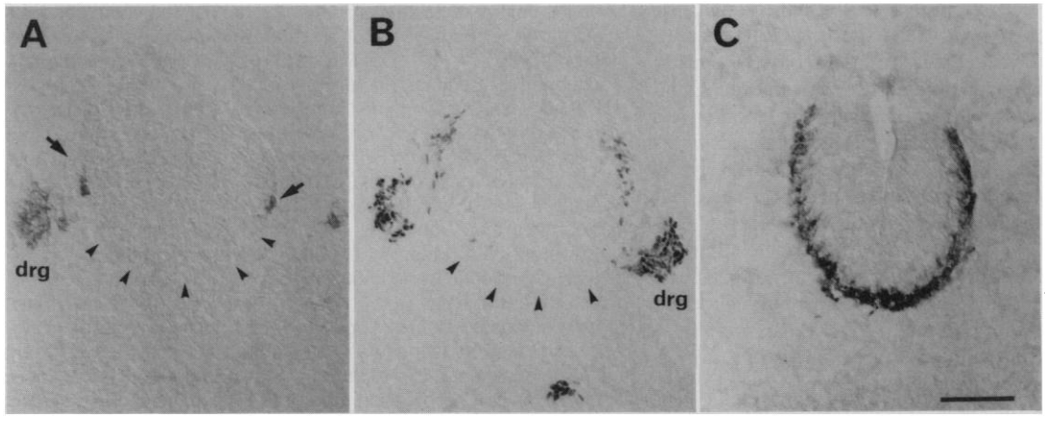


**Fig. 4.** Islet-1 is not expressed by cells in the ventral spinal cord in the absence of the notochord and floor plate. The notochord was removed from the caudal neural tube of stage 9 to 10 chick embryos, which were then incubated for 48 hours, until stage 29. (**A**) The SC1 antigen is not expressed by cells within the spinal cord. The two ventral patches of SC1-labeling (arrows) correspond to sensory axons in the dorsal root entry zone, which is located in an abnormally ventral position. (**B**) Section of spinal cord showing that Islet-1 is not expressed by cells in the ventral region. Note that a subset of cells in the dorsal spinal cord still expresses Islet-1 but that these cells do not express SC1 (A). Cells in the dorsal root ganglia express both Islet-1 and SC1. In (A) and (B) arrowheads demonstrate the ventral border of the spinal cord. (**C**) An adjacent section from the same embryo shows CRABP is expressed by cells around the perimeter of the spinal cord including the extreme ventral region. This antigen is normally restricted to cells in dorsal and intermediate regions of the spinal cord (*5*). Scale bar: (A to C) 60 μm.

notochord or floor plate grafts (Fig. 3A) appears to result from the induction of motor neurons and not from the proliferation and precocious differentiation of Islet-1–positive cells that are normally present in the dorsal spinal cord. These results provide evidence that signals from the notochord and floor plate can induce the expression of Islet-1 in dorsal neural tube cells.

Elimination of the notochord and floor plate before neural tube closure results in the development of a spinal cord that is devoid of ventral neuronal types including motor neurons (*4*, *5*). To examine whether Islet-1 expression in the ventral spinal cord is dependent on signals from the notochord and floor plate, we removed the notochord from the caudal region of stage 9 to 10 chick embryos, thus preventing floor plate differentiation (*25*). In the notochord- and floor plate–free regions of such embryos, Islet-1 and SC1 were not expressed in ventral spinal cord cells at any period from stages 15 to 29 (Fig. 4, A and B). These results support the idea that elimination of the notochord and floor plate prevents the initial steps in the differentiation of motor neurons. However, our results do not exclude the possibility that the specification of motor neurons is initiated before the elimination of the notochord and floor plate and that the absence of Islet-1 and other later markers reflects an arrest in the differentiation of motor neurons at a stage before the expression of Islet-1.

In the spinal cord of embryos from which the notochord and floor plate have been eliminated, several markers of dorsal and intermediate cell types such as AC4 (*4*) and a cellular retinoic acid–binding protein (CRABP) (*5*) appear at the ventral midline, suggesting that their dorsal restriction in normal embryos is conferred by signals from the notochord and floor plate that repress expression in ventral regions. However, the differentiation of cells in the dorsal spinal cord could require inductive signals, one source of which may be roof plate cells, which are located at the dorsal midline of the spinal cord (*5*). After notochord removal, some Islet-1–positive cells remain in the spinal cord (Fig. 4B), but these are restricted to dorsal and intermediate regions in a pattern similar to that observed in unoperated embryos. These residual Islet-1–positive cells may correspond to the dorsolateral Islet-1–positive cells observed in normal embryos. The restriction of these Islet-1–positive cells to dorsal regions contrasts with the ventral position of cells expressing CRABP (Fig. 4C) and AC4 after notochord removal and supports the idea that signals distinct from those provided by the notochord and floor plate are required for the differentiation of this dorsal cell group.

In conjunction with other recent studies, the present results provide preliminary information on the early stages of differentiation of spinal motor neurons. Signals from the notochord and floor plate appear to induce uncommitted neural progenitors to acquire motor neuron fates (*2*–*5*). The earliest identified response of presumptive motor neurons to these inductive signals is the expression of the homeobox gene *Islet-1*, which delineates the entire population of spinal motor neurons, both somatic and visceral. This initial set of motor neurons gives rise to distinct subtypes, which can be identified by their axonal projection patterns and functional properties. Studies in zebrafish embryos have provided evidence that the determination of subsets of motor neurons, defined by their axonal projection patterns, occurs shortly after that of the motor neuron population as a whole (*26*).

Homeobox genes are involved in many aspects of vertebrate development (*27*). The pattern of expression of *Hox* genes along the anterior-posterior axis (*28*) and of *Pax* genes along the dorsoventral axis of the neural tube (*29*), together with the phenotypes that result from inactivation of some of these genes (*30*), suggests that they contribute to the regional patterning of the developing nervous system. In contrast, the restricted expression of Islet-1 and the involvement of related LIM-homeodomain proteins in the determination of cell fate in *C. elegans* (*8*, *9*) suggest that Islet-1 may be involved in specifying the fate of specific neuronal subtypes, including motor neurons.

## REFERENCES AND NOTES


1. C. Lance-Jones and L. Landmesser, *Proc. R. Soc. London Ser. B* **214**, 1 (1981); E. Frank and G. D. Fischbach, *J. Cell Biol.* **83**, 143 (1979); U. J. McMahan, *Cold Spring Harbor Symp. Quant. Biol.* **55**, 407 (1990); V. Hamburger and R. W. Oppenheim, *Neurosci. Comment.* **1**, 39 (1982).
2. S. M. Leber, S. M. Breedlove, J. R. Sanes, *J. Neurosci.* **10**, 2451 (1990).
3. H. W. M. van Straaten, F. Thors, E. L. Wiertz-Hoessels, J. W. M. Hekking, J. Drukker, *Dev. Biol.* **110**, 247 (1985).
4. T. Yamada, M. Placzek, H. Tanaka, J. Dodd, T. M. Jessell, *Cell* **64**, 635 (1991).
5. M. Placzek, T. Yamada, M. Tessier-Lavigne, T. M. Jessell, J. Dodd, *Development Suppl.* **2**, 105 (1991).
6. O. Karlsson, S. Thor, T. Norberg, H. Ohlsson, T. Edlund, *Nature* **344**, 879 (1990).
7. M. P. Scott, J. W. Tamkun, G. W. Hartzell, *Biochem. Biophys. Acta* **989**, 25 (1989).
8. J. C. Way and M. Chalfie, *Cell* **54**, 5 (1988).
9. G. Freyd, S. K. Kim, H. R. Horvitz, *Nature* **344**, 876 (1990).
10. J. Ericson, S. Thor, T. Brannstrom, T. Edlund, *Neuron* **7**, 881 (1991).
11. Chick embryos were staged according to Hamburger and Hamilton (*12*). Embryos were fixed in 4% paraformaldehyde (*4*) and cryostat sections (10-μm thickness) were stained with rabbit antiserum to Islet-1 diluted at 1:500 and HRP-conjugated goat antibody to rabbit immunoglobulin G (IgG). The Islet-1 antibody used in this study was raised against a rat Islet-1 fusion protein (*10*). The antibody detects Islet-1 immunoreactivity in all vertebrate species that have been examined (J. Ericson, S. Thor, T. Edlund, unpublished data). The pattern of expression of Islet-1 in neural tissue observed in this report is similar to that observed in rat, mouse, *Xenopus*, and zebrafish embryos.
12. V. Hamburger and H. Hamilton, *J. Morphol.* **88**, 49 (1951).
13. Cell numbers were determined on 10-μm cryostat sections stained with antibodies to Islet-1 and HRP-conjugated antibody to rabbit immunoglobulin. At stages 28 to 29 the number of the Islet-1–positive cells (Table 1A) was somewhat larger than the number of motor neurons reported in other studies [R. Oppenheim *et al.*, *Dev. Biol.* **133**, 468 (1990)] in which motor neurons were defined by their morphology and ventrolateral location in the spinal cord. However, determination of the number of Islet-1–positive cells was performed at stages when it is difficult to identify motor neurons solely by morphology, making direct comparison of numbers problematic.
14. M. Hollyday and V. Hamburger, *Brain Res.* **132**, 197 (1977).
15. The ventral surface of the spinal cord was exposed by a ventral laminectomy and recrystallized HRP (50% w/v, Sigma grade IV) was applied to the cut end of the ventral roots. Embryos were then incubated in L-15 for 4 to 6 hours at 37°C to allow retrograde transport of HRP. The 10-μm cryostat sections were incubated with mouse monoclonal antibody to HRP (Sigma, St. Louis, MO) and rabbit antiserum to Islet-1 (*10*) at 4°C overnight. After washing, species-specific secondary antibodies conjugated with different fluorescent dyes were applied. The immunostained sections were mounted with glycerol containing 1 mg of paraphenylenediamine (PPDA) per milliliter to prevent bleaching.
16. R. Levi-Montalcini, *J. Morphol.* **86**, 253 (1950); R. Oppenheim *et al.*, *J. Comp. Neurol.* **210**, 174 (1950).
17. J. Langman and C. C. Haden, *J. Comp. Neurol.* **138**, 419 (1970); R. W. Oppenheim *et al.*, *ibid.* **275**, 159 (1988); E. M. Carpenter and M. Hollyday, *Dev. Biol.* **150**, 144 (1992).
18. H. Tanaka and K. Obata, *Dev. Biol.* **106**, 26 (1984); H. Tanaka *et al.*, *Neuron* 7, 535 (1991); E. Bloch-Gallego *et al.*, *Development* **111**, 221 (1991). We double-labeled with antibodies to Islet-1 and SC1 by incubating sections of stage 15 to 17 brachial spinal cord with the two primary antibodies overnight at 4°C and then with species-specific secondary antibodies (*15*). The sections were mounted with glycerol-PPDA and examined with a confocal laser microscope (Bio-Rad, Richmond, CA) with an optical section of 1 to 2 μm. SC1 labels the notochord and floor plate in addition to motor neurons.
19. S. K. McConnell and C. E. Kaznowski, *Science* **254**, 282 (1991).
20. J. Langman and C. C. Haden, *J. Comp. Neurol.* **138**, 419 (1970).
21. BrdU (100 to 200 μl, 100 μM in L-15 medium) (Specialty Media Inc.) was applied directly onto stage 15 to 16 chick embryos in ovo. The 10-μm cryostat sections were processed first with antibody to Islet-1 and Texas Red–conjugated goat antibody to rabbit IgG. The Islet-1–labeled sections were then treated with 2 N HCl for 15 to 20 min at room temperature and washed with 0.1 M phosphate buffer. Mouse monoclonal antibody to BrdU (Becton Dickenson, Mountain View, CA) was applied (1:20 dilution in 10 mM phosphate-buffered saline (PBS) containing 0.1% Triton X-100) for 30 min at room temperature and incubated with fluorescein isothiocyanate (FITC)–conjugated goat antibody to mouse IgG. Double-labeled sections were analyzed by confocal laser microscopy.
22. J. L. Smith and G. C. Schoenwolf, *Cell Tissue Res.* **252**, 491 (1988); J. Langman, R. L. Guerrant, B. G. Freeman, *J. Comp. Neurol.* **127**, 399 (1966).
23. This series of experiments did not rule out that cells that had begun to express Islet-1 underwent further rounds of cell division and that BrdU

persists at detectable concentrations in the nuclei of daughter cells. To examine this possibility, we performed similar experiments but applied BrdU about 12 hours later at stages 17 to 18, during the peak of motor neuron generation (*14*). Again, few (<1%) Islet-1–positive cells had incorporated BrdU within 4 hours (Table 2). If the cells that had begun to express Islet-1 at stages 15 to 16 underwent further rounds of cell division, then this should have been reflected by the rapid (<4 hours) incorporation of BrdU into Islet-1–positive cells at stages 17 to 18, which was not observed (Table 2). These results reinforce the conclusion that Islet-1 is expressed only in postmitotic motor neurons. The small percentage of cells that coexpress Islet-1 and BrdU within 4 hours may reflect cells that are labeled at late stages of S phase.

24. Notochords from stage 10 and floor plate tissue from stages 17 to 18 chick embryos were grafted adjacent to the caudal neural tube of stage 10 hosts (*4*). Approximately 48 hours after grafting, the embryos were fixed and serial sections were stained with antibodies to Islet-1, SC1, and FP1.
25. A segment of notochord was removed from the caudal region of the neural tube of stage 9 to 10 chick embryos [M. Placzek, M. Tessier-Lavigne, T. Yamada, T. Jessell, J. Dodd, *Science* **250**, 985 (1990); also (*4*)]. Approximately 48 hours after removal of the notochord, the embryos were fixed and serial sections stained with antibodies to Islet-1, SC1, CRABP, AC4, and FP1 (*4, 5*).
26. J. S. Eisen, *Science* **252**, 569 (1991).
27. W. McGinnis and R. Krumlauf, *Cell* **68**, 283 (1992).
28. D. Duboule and P. Dolle, *EMBO J.* **8**, 1497 (1989); A. Graham, N. Papalopulu, R. Krumlauf, *Cell* **57**, 367 (1989); P. Hunt *et al.*, *Nature* **353**, 861 (1991).
29. M. D. Goulding *et al.*, *EMBO J.* **10**, 1135 (1991); C. Walther and P. Gruss, *Development* **113**, 1435 (1991).
30. O. Chisaka and M. R. Capecchi, *Nature* **350**, 473 (1991); T. Lufkin, A. Dierich, M. Lemeur, M. Mark, P. Chambon, *Cell* **66**, 1105 (1991); D. J. Epstein, M. Vekemons, P. Gros, *ibid.* **67**, 767 (1991); C. C. Ton *et al.*, *ibid.*, p. 1059; R. E. Hill *et al.*, *Nature* **354**, 522 (1991).
31. We thank J. Dodd, A. Furley, M. Placzek, and A. Ruiz i Altaba for comments on the manuscript and V. Leon for secretarial assistance. We also thank E. Carpenter for communicating results before publication and L. Role for discussion. This work was supported, in part, by grants from the Swedish National Science Research Council, Swedish Medical Research Council, Swedish National Board for Technical Development, and the Juvenile Diabetes Foundation, New York, to T.E. T.Y. is an associate and T.M.J. is an investigator of the Howard Hughes Medical Institute.

4 February 1992; accepted 14 April 1992

# Galactose Oxidation in the Design of Immunogenic Vaccines

Biao Zheng, Sara J. Brett, John P. Tite, M. Robert Lifely, Thomas A. Brodie, John Rhodes*

**Potent immunological adjuvants are urgently required to complement recombinant and synthetic vaccines. However, it has not been possible to derive new principles for the design of vaccine adjuvants from knowledge of the mechanism of immunogenicity. Carbonyl-amino condensations, which are essential to the inductive interaction between antigen-presenting cells and T helper cells, were tested as a target for the enhancement of immune responses. Enzymic oxidation of cell-surface galactose to increase amine-reactive carbonyl groups on murine lymphocytes and antigen–presenting cells provided a potent, noninflammatory method of enhancing the immunogenicity of viral, bacterial, and protozoal subunit vaccines in mice.**

Vaccination, the most effective means of combatting infectious disease, requires the use of adjuvants that nonspecifically enhance the immunogenicity of attenuated, killed, or fragmented microorganisms. This is particularly important for recombinant proteins and synthetic peptides, which are substantially less immunogenic than conventional vaccines. Aluminum salts (alum), first described as adjuvants more than 60 years ago (*1*), remain the only agents approved for human use. Their potency is limited and they do not consistently enhance cell-mediated responses likely to be important in antiviral immunity. New vaccine adjuvants and vehicles at the preclinical research stage include surface-active agents, liposomes, immune-stimulating complexes (ISCOMS), and adjuvant peptides (*2*). These approaches seek to mimic general properties of infectious pathogens such as persistence, size, lipophilicity, and the ability to stimulate macrophages nonspecifically. Alternatively, live attenuated vectors have been developed that, because of their ability to productively infect a susceptible host, confer immunogenicity on the recombinant antigens they carry (*3*). In contrast to these approaches, we have targeted covalent chemical events between cellular ligands that appear to be essential to immune induction.

The primary specific event in most immune responses is the inductive interaction between antigen (Ag)–presenting cells (APC) and T helper lymphocytes ($T_H$ cells) (*4*). In addition to the recognition that takes place between the T cell receptor and the complex of Ag and major histocompatibility complex (MHC) class II molecule, essential accessory interactions occur at the macromolecular level (*5*). At the chemical level, transient carbonyl-amino condensation (Schiff base formation) between cell-surface ligands also appears to be essential for Ag-specific T cell activation (*6*). This reaction might provide a means of enhancing responses to potential vaccines by increasing the expression of reactive ligands. To test this idea, we used galactose

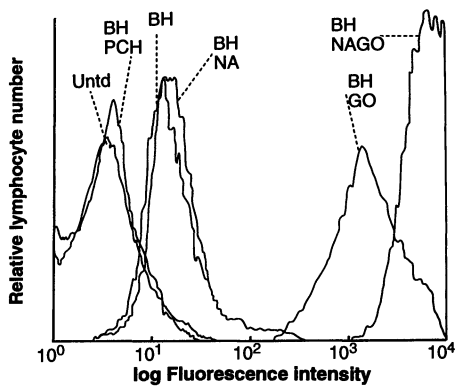


**Fig. 1.** Flow cytofluorometric measurement of amine-reactive carbonyl groups on the lymphocyte surface. Freshly prepared murine (BALB/c) spleen cells ($5 \times 10^6$/ml in PBS) were treated with biotin hydrazide (Sigma, Poole, Dorset, United Kingdom) (5 mM, 1 hour, 37°C), which forms a covalent hydrazone (C = N) linkage with reactive carbonyl groups. This reversible bond was then reduced with $NaCNBH_3$ at neutral pH (10 mM, 1 hour, 20°C). After the reduction, cells were washed, treated with fluorescein isothyocyanate (FITC)–avidin (Vector Laboratories, Peterborough, United Kingdom) (1:50 dilution) (for 30 min at 4°C), and washed three times. Flow cytofluorometric measurements (gated on lymphocytes) were obtained with a FACScan (Becton Dickinson), with the use of Consort 30 data management. Pyridinium chloride hydrazide (Sigma) treatment was at 10 mM for 1 hour at 37°C, followed by reduction with $NaCNBH_3$. Cells were exposed to neuraminidase at 2.5 U/ml for 30 min at 37°C and washed. Treatment with GO (5 U/ml) and with NAGO (NA 2.5 U/ml; GO 5 U/ml) was performed in the same way. Lymphocytes were exposed to the following: FITC-avidin only (Untd); pyridinium chloride hydrazide and then to biotin hydrazide (BH PCH); biotin hydrazide (BH); NA and then to biotin hydrazide (BH NA); GO and then to biotin hydrazide (BH GO); and NAGO and then to biotin hydrazide (BH NAGO).

B. Zheng and J. Rhodes, Department of Pharmacology, Wellcome Research Laboratories, Langley Court, South Eden Park Road, Beckenham, Kent, United Kingdom BR3 3BS.
S. J. Brett, J. P. Tite, M. R. Lifely, Department of Cell Biology, Wellcome Research Laboratories, Beckenham, Kent, United Kingdom BR3 3BS.
T. A. Brodie, Department of Drug Safety Evaluation, Wellcome Research Laboratories, Beckenham, Kent, United Kingdom BR3 3BS.

*To whom correspondence should be addressed.