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10. We have sampled 100 crickets from nine populations, ranging from pure *G. pennsylvanicus* populations in the northwest hills of Connecticut to pure *G. firmus* along the coast.
11. Total DNA was isolated from individual crickets by a method developed for *Drosophila* in the laboratory of David Hogness (S. Artavanis-Tsakonas, personal communication). Crickets were ground in liquid nitrogen and then homogenized in 5 ml of buffer in the presence of DEPC. The homogenate was incubated at 70°C for 30 minutes, 0.75 ml of 8*M* potassium acetate was added and the mixture was put on ice for an hour. The resulting precipitate was spun down and two volumes of ethanol were added to the supernatant to precipitate DNA. The DNA was pelleted and the pellet was resuspended in TE buffer and treated with ribonuclease. The DNA was then extracted with phenol, phenol:chloroform, and chloroform and precipitated with ethanol.
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13. *Grylus assimilis* is a close relative of *G. pennsylvanicus* and *G. firmus* and was used as a source of pure mtDNA for two reasons: (i) *G. assimilis* has continuous generations in the laboratory (no diapause) and single isofemale lines can be reared conveniently; (ii) in contrast to *G. pennsylvanicus* and *G. firmus*, it is a long-winged species with substantial flight muscles that are a rich source of mitochondria. To obtain pure mtDNA, we homogenized thoracic muscle from 25 to 50 adult crickets and separated mitochondria by differential centrifugation. The mitochondria were lysed with SDS, and mtDNA was purified away from residual nuclear DNA contamination by equilibrium centrifugation in CsCl–propidium diiodide.
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15. The label designates the fragment patterns for the enzymes Apa I, Hinc II, Hind III, and Xba I. The AAAA genotype has the A fragment pattern for each enzyme.
16. One enzyme, Bgl II, did not show any fragment pattern differences among individuals with mtDNA's of different sizes. Moreover, in all digests the sum of the Bgl II fragments was about 1.0 kb less than the sum of the fragments produced by other enzymes. Mapping studies indicate a "missing" 1.2-kb Bgl II fragment which does not show up on our autoradiographs, presumably because no similar sequence occurs in *G. assimilis* mtDNA. Only in Bgl II digests is a fragment produced that falls entirely within the nonhomologous region. We suggest that this rapidly evolving sequence also contains the region of addition or deletion in *G. pennsylvanicus* and *G. firmus*.
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Selective Inhibition of Fibronectin-Mediated Cell Adhesion by Monoclonal Antibodies to a Cell-Surface Glycoprotein

Abstract. *Fibroblasts possess several distinct mechanisms that control cellular adhesion to extracellular matrix macromolecules. Monoclonal antibodies to a 140-kilodalton (kD) cell surface glycoprotein inhibited the adhesion of fibroblastic Chinese hamster ovary cells to fibronectin-coated substrata but did not inhibit adhesion to substrata coated with vitronectin, laminin, serum, or other adhesive macromolecules. Thus the 140-kD glycoprotein appears to be involved in the fibronectin-mediated adhesion mechanism but not in other adhesion processes.*

Adhesive interactions between cells and components of the extracellular matrix are important in cell growth and differentiation (*1*), in morphogenetic processes during ontogeny (*2*), and in the metastases of malignant cells (*3*). Progress has been made toward elucidating the structure and function of molecules of the extracellular matrix (such as fibronectin and laminin) that are involved in cell adhesion (*4*). In contrast, there has been relatively little progress in identifying and characterizing plasma membrane components involved in adhesion. A candidate molecule for the cellular receptor for laminin has been identified (*5*); recent identification of a short peptide with adhesion-promoting activity suggests the existence of a specific surface receptor for fibronectin (*6*). However, although gangliosides (*7*), glycosaminoglycans (*8*), and glycoproteins (*9*) have each been suggested as the cellular fibronectin receptor, there is little evidence supporting any of these alternatives. Both large [120 to 160 kilodaltons (kD)] and small (40 to 60 kD) surface glycoproteins have been proposed as the receptor (*10, 11*). We have now developed a monoclonal antibody that inhibits the adhesion of hamster fibroblasts to fibronectin-coated substrata but does not inhibit adhesion to substrata coated with other ligands. The antibody immunoprecipitates a cell surface glycoprotein of approximately 140 kD; this molecule appears to play a central role in fibronectin-mediated adhesion.

Fibroblastic Chinese hamster ovary (CHO) cells were maintained in suspension culture (*12*). These cells have both fibronectin-dependent (type I) and fibronectin-independent (type II) adhesion mechanisms (*12, 13*) and readily adhere to substrata that have been coated with a variety of ligands, including fibronectin and its adhesive fragments, vitronectin, laminin, serum, lectins, and extracellular matrix material (*12–16*).

To isolate monoclonal antibodies to CHO cell surface proteins, we repetitively immunized BALB/c mice with CHO cells that had been trypsinized gently (20 μg/ml, 10 minutes at 25°C) and emulsified in Freund's complete adjuvant. Spleen cells from immunized mice were fused with P3x63-Ag8 myeloma cells, and hybridomas were isolated (*17*). Supernatants of hybridoma cultures were screened either for ability to bind to CHO cell surfaces by means of an enzyme-linked immunosorbent assay (ELISA) (*18*) or for ability to impede CHO cell adhesion to microwells coated with fibronectin. Of 8448 supernatants screened, 193 manifested cell-binding activity while only two displayed adhesion-blocking activity. Selected hybridoma cultures were cloned (*18*); cloned cells were grown in ascites form in pristane-primed BALB/c mice. The serotype of the ascites immunoglobulin was determined with commercial reagents. The antibody was then purified by ammonium sulfate precipitation and ion exchange chromatography (*19*) to approximately 95 percent homogeneity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Techniques for the preparation of ligand-coated substrata and for measuring the adhesion of isotopically labeled CHO cells to these substrata have been described (*12, 13*), as are procedures for surface or metabolic labeling of CHO cell membrane proteins (*20*). Immunoprecipitations of nonionic detergent-solubilized membrane proteins were carried out with monoclonal immunoglobulin G (IgG) linked, via a rabbit antibody to mouse globulin, to protein A–Sepharose (Pharmacia) (*21*) or with monoclonal antibody covalently linked to Affigel (Bio-Rad).

Most monoclonal IgG's directed against CHO cell surface components do not affect cell adhesion. However, two clones produced IgG's (PB1 and PB2; subclass IgG_1) capable of effectively blocking adhesion to fibronectin at concentrations of approximately 1 μg/ml (Fig. 1). By contrast, antibodies produced by two other clones (3F9 and 6C10), although directed against abundant surface proteins of 265 kD and 100 kD, respectively, did not block adhesion

at concentrations in excess of 100 μg/ml. Treatment of cells with 3F9 or 6C10 followed by rabbit antibody to mouse IgG, which should lead to cross-linking and "patching" (*22*) of the 3F9 or 6C10 antigens, also did not affect adhesion. Thus, a restricted set of cell surface components appears to be crucially involved in the fibronectin-mediated cell adhesion process. No toxicity was associated with the inhibition of adhesion caused by PB1 or PB2; the cells continued to exclude trypan blue and, when washed free of antibody, grew at a normal rate in suspension. PB1 seems to have been more potent than PB2 of equal purity, but it is not clear if this represents a difference in antibody affinity or simply a higher fraction of biologically active antibody. Initial binding studies with ^{125}I-labeled antibodies indicated that there are approximately 10^5 PB1 antigen sites per cell, that the affinity of PB1 for its binding site is about $1 \times 10^{-9} M$ and that PB1 and PB2 compete for binding sites.

PB1 or PB2 were able to block adhesion to substrata coated with intact fibronectin or with F105, a 105-kD adhesion-promoting chymotrypic fragment of fibronectin that lacks binding sites for gelatin and heparin (*14, 23*) (Fig. 2). PB1 and PB2 did not affect CHO cell adhesion to substrata coated with vitronectin, laminin, or serum. The antibodies 3F9 and 6C10 did not block adhesion to any of the substrata, although 3F9 seemed to enhance adhesion to a small extent in some cases. In these assays approximately 40 percent of the CHO cells adhered to the laminin substratum while adhesion to the other substrata ranged from 85 to 95 percent (*16*). Neither PB1 or PB2 could block CHO cell adhesion to lectin-coated substrata. Thus, these antibodies selectively block the fibronectin-mediated adhesion process but do not affect processes mediated by other adhesive proteins.

We also examined the effect of PB1 on the type II (fibronectin-independent) adhesion mechanism found in CHO cells (*13*). This mechanism involves attachment of cells to extracellular matrix material and is demonstrated by adhesion of AD^{v}F11 cells (a variant clone of CHO that lacks the fibronectin-mediated adhesion mechanism), or by adhesion of wild-type cells after blocking fibronectin in the matrix with antibody to fibronectin. Amounts of PB1 that completely blocked adhesion of wild-type cells to fibronectin produced only a small decrease in type II adhesion of wild-type or AD^{v}F11 cells (Table 1). Thus, the PB1 monoclonal antibody blocks fibronectin-dependent adhesion (type I) at doses that do not block type II adhesion.

Immunoprecipitation of CHO cells surface-labeled with ^{125}I (*24*), followed by SDS-PAGE and autoradiography (*13*), revealed that PB1 and PB2 both precipitate a surface component of 140-kD (Fig. 3A). This component is distinct from the major surface complex (120 to 140 kD) seen in the whole-cell lysate. The 140-kD component was also the predominant species in immunoprecipitates from cells labeled with [^{35}S]methionine or [^{3}H]glucosamine (Fig. 3A), indicating that it is a cell-derived surface glycoprotein. A number of other components (20 to 50 kD) found in immunoprecipitates of [^{35}S]methionine-labeled lysates were also observed in control preparations and therefore are precipitation artifacts. Analyses on highly resolving 5 percent gels under reducing conditions also showed the 140-kD glycoprotein to be a single component (Fig. 3B). The 140-kD component was the only component specifically precipitated by our adhesion-blocking monoclonal antibodies from cells labeled with ^{125}I, [^{35}S]methionine, or [^{3}H]glucosamine. Conversely, the 140-kD component was not precipitated by the non-blocking monoclonal antibodies 6C10

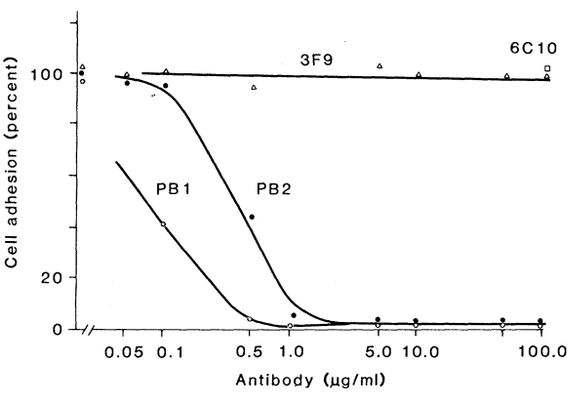

Fig. 1. Inhibition of CHO cell adhesion to fibronectin substrata by monoclonal antibodies. Affinity-purified (*14*) bovine serum fibronectin (40 μg/ml) was adsorbed onto gelatin-coated 24-well tissue culture plates for 2 hours; the wells were coated with 1 percent bovine serum albumin (BSA) for an additional 30 minutes and then rinsed with isotonic phosphate buffer, *p*H 7.2 (PBS). Dilutions of monoclonal IgG's were made in ice-cold αMEM (Gibco) containing 0.1 percent BSA and added to the wells. Washed CHO cells, previously labeled with [^{3}H]leucine overnight in suspension culture, were added to the antibody-containing wells at a concentration of 5×10^4 cells per well. The plates were incubated for 90 minutes at 0°C and a further 90 minutes at 37°C. The nonadherent cells were removed by washing with PBS, and radioactivity in the adherent cells was measured by solubilizing cells in 1 percent SDS, suspending in Aquasol (NEN), and counting in a scintillation counter. Each point represents the mean of three determinations, which differed by less than 10 percent. Antibodies PB1 and PB2 are not directed against fibronectin. Thus, they neither precipitated soluble fibronectin nor reacted with substratum-bound fibronectin in an ELISA assay; moreover, preincubation of the substratum with antibody, followed by washing, did not result in blockage of cell adhesion, whereas preincubation of cells with antibody, followed by washing, did block adhesion. Data are expressed as the percent of adhesion relative to the control (no antibody).

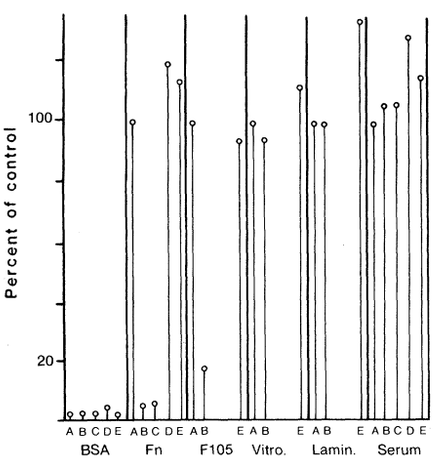

Fig. 2. Selectivity of adhesion inhibition by monoclonal antibodies. Ligands were incubated in 24-well tissue culture plates for 1 to 2 hours at 37°C. Excess ligand was removed, and then the wells were coated with 1 percent BSA; in the case of laminin the excess ligand was allowed to remain, as laminin binds well to cells but not to tissue-culture plastic (*16*). The following substrata were used: Fn, affinity-purified bovine fibronectin (40 μg/ml); F105, the adhesion-promoting domain of fibronectin (40 μg/ml); vitro, vitronectin (40 μg/ml); lamin, laminin (60 μg/ml); serum, fetal bovine serum (10 percent). Isotopically labeled CHO cells (10^6/ml) were incubated with monoclonal IgG's (100 μg/ml) in αMEM + 0.1 percent BSA or in αMEM + 0.1 percent BSA without antibody for 90 minutes at 0°C. Then, 50 μl of cell suspension was added to 1 ml of αMEM + 0.1 percent BSA in the ligand-coated wells, and incubation was continued for 90 minutes at 37°C. Nonadherent cells were washed away and the residual adherent cells were counted as in Fig. 1. Results are the means of triplicate determinations. Ordinate, percent of adhesion relative to the untreated control; abscissa, substratum type. For BSA (nonadherent controls) the data represent the percent of total cells that adhered. A, control; B, PB1 (100 μg/ml); C, PB2 (100 μg/ml); D, 3F9 (100 μg/ml); E, 6C10 (100 μg/ml).

and 3F9, which instead precipitate components of 100-kD and 265-kD, respectively. Thus, the 140-kD surface glycoprotein seems to be the key component in the blocking of fibronectin-mediated adhesion caused by PB1 or PB2.

Fibroblasts can adhere to the extracellular matrix by several distinct mechanisms (*1, 13*). The 140-kD cell surface glycoprotein defined by monoclonal antibodies PB1 and PB2 seems to play a key role in fibronectin-mediated cell adhesion but not in processes mediated by other adhesive proteins. Immunological approaches to the study of cell-substratum adhesion have also been used by other investigators (*25–30*).

However, in the previous reports, the antibodies used blocked adhesion to complex substrata (such as serum-coated tissue culture dishes) which are likely to have several adhesion-promoting ligands (*1, 14, 15*). Thus, simultaneous actions on several distinct adhesion mechanisms may have occurred. It is also possible that polyclonal antibodies may (by cross-linking) have perturbed membrane or cytoskeletal processes and thus produced an indirect and general modulation of adhesion. The monoclonal antibodies PB1 and PB2 selectively inhibited fibronectin-mediated adhesion but did not affect adhesion events mediated by other ligands. This suggests that effects on multiple adhesion mechanisms or nonspecific perturbations of membrane or cytoskeletal processes are unlikely. PB1 and PB2 immunoprecipitated a 140-kd glycoprotein which, although usually appearing as a single component, sometimes appeared as a doublet in experiments utilizing highly resolving 5 percent gels under nonreducing conditions. This glycoprotein may resemble other adhesion proteins which show altered SDS-gel mobilities in the absence of reducing agents (*30*). The 140-kD glycoprotein (or glycoproteins) defined by monoclonal antibodies PB1 and PB2 may be a member of the family of surface glycoproteins previously defined in mammalian cells with a polyclonal antiserum (*30*); the 140-kD glycoprotein may also resemble the complex of adhesion proteins identified in avian cells (*27, 29*). However, in contrast to other antibodies that block adhesion to multiple ligands, PB1 and PB2 were specific for the fibronectin-mediated process.

It is unclear whether the 140-kd glycoprotein is truly a "receptor" or binding site for fibronectin or an accessory-coupling protein that acts with the receptor in the fibronectin-mediated adhesion pathway. The concept of a two-step process, involving an entity that can bind fibronectin (a ganglioside for example), which then interacts with a coupling protein to transduce the "signal" for adhesion, is an attractive one and has many parallels in peptide hormone-receptor systems (*31*). Nonetheless, at present it is not possible to assign a definite role to the 140-kD glycoprotein, but only to state that it is specific for and critical to the fibronectin-mediated adhesion process.

Table 1. Lack of effect of PB1 on percent of type II adhesion. Tissue culture substrata coated with extracellular matrix material from human fibroblasts (SAM, substrate attached material) were prepared as described (*13*). Wild-type (WT) and adhesion-variant (AD^{v}F11) CHO cells were labeled with [^{3}H]leucine and allowed to attach to SAM- or fibronectin (Fn)-coated substrata for 90 minutes at 37°C in αMEM plus 0.1 percent BSA. The PB1 was included in the adhesion assay buffer for a 90-minute incubation at 4°C prior to allowing adhesion to proceed at 37°C. In some cases, SAM or Fn substrata were treated with a 1/200 dilution of monospecific rabbit antibody to bovine fibronectin (anti-Fn) before the assay. Results are the means and standard errors of triplicate determinations normalized on the basis of the adhesion of untreated (no PB1 antibody) controls. The adhesion of the untreated cells to SAM or anti-Fn treated SAM was 30 percent (AD^{v}F11) to 60 percent (WT) while adhesion of WT cells to Fn was approximately 80 percent. AD^{v}F11 cells do not adhere to Fn (*13*). There was only 5 ± 1.0 percent adhesion of WT cells on fibronectin-coated plates in the presence of anti-Fn.

Substratum	Percent of type II adhesion at PB1 concentrations (μg/ml)				
	0	0.01	0.1	1.0	10.0
			WT cells		
SAM + anti-Fn	100	93.3 ± 1.7	85.5 ± 7.3	78.5 ± 2.4	79.5 ± 4.1
SAM	100	112.8 ± 2.2	103.8 ± 1.6	91.2 ± 3.4	69.7 ± 6.8
Fn	100	98.8 ± 0.6	98.1 ± 1.6	56.8 ± 6.3	11.0 ± 1.3
			Ad^{v}F11 cells		
SAM	100		90.8 ± 5.5		83.3 ± 5.0

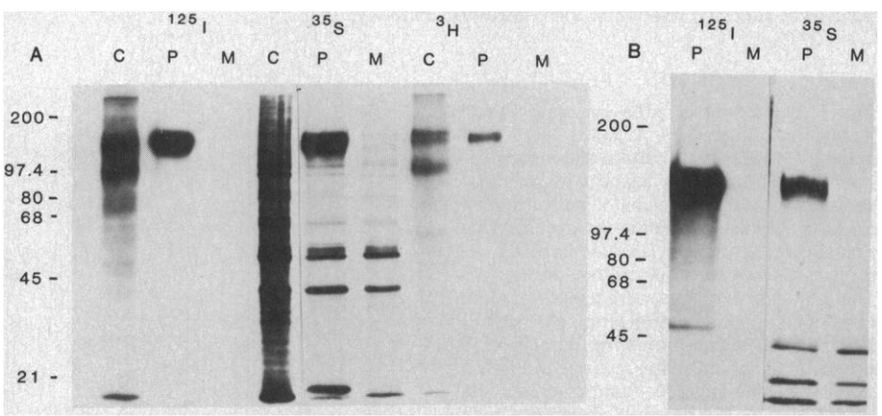

Fig. 3. Immunoprecipitation of surface-labeled and metabolically labeled cell lysates with adhesion-blocking monoclonal antibodies. Exponentially growing CHO cells were surface-labeled with ^{125}I by means of 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodogen; Pierce Chemical Co.) or, alternatively, were metabolically labeled overnight with [^{35}S]methionine (10 μCi/ml) or [^{3}H]glucosamine (5 μCi/ml) as described (*13, 32*). Cells were lysed in 1 percent Nonidet P-40 + 1 m*M* phenylmethylsulfonyl fluoride and were subjected to immunoprecipitation with either specific monoclonal antibody (PB2) or nonspecific mouse IgG (MGG) coupled to Affigel (Bio-Rad). Samples were analyzed by SDS-PAGE and autoradiography (*13*). The immunoprecipitation patterns for PB1 were identical to those of PB2. Marker size is shown in kilodaltons. C, whole-cell lysate; P, specific immunoprecipitate (PB2); M, nonspecific control precipitate (MGG). (A) 7.5 percent acrylamide; (B) 5.0 percent acrylamide [gel includes 0.3 percent linear polyacrylamide for enhanced resolution (*13*)].

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Morphine-Induced Delay of Normal Cell Death in the Avian Ciliary Ganglion

Abstract. *Repeated administration of morphine in increasing doses delayed normal cell death in the ciliary ganglion of the chick embryo; the effect was completely blocked by naloxone. Survival of spinal motoneurons was not affected. Morphine also inhibited potassium-stimulated synthesis of acetylcholine in ganglion cells cocultured with muscle, suggesting that morphine can influence neurotransmission. Morphine's effect on cell death may be due to an inhibition of transmission at the neuromuscular junction, but opiates may also directly affect cell death. Although it is not known whether the endogenous opiates in the ciliary ganglion influence neuronal survival during embryogenesis, exogenous opiates can affect normal cell death in the autonomic nervous system.*

Neuronal death during development occurs naturally throughout the nervous system (*1*) and can be influenced by muscular activity (*2*), the target, and the trophic support (*3, 4*). In the avian ciliary ganglion, cells die during the period of synapse formation with the target (*5*). Ciliary ganglion cells project to the muscles of the iris, ciliary body, and choroid coat. Normal cell death in this system results in a reduction from 6260 ± 339 cells (mean ± standard error) on embryonic day 8 to 3704 ± 241 cells on embryonic day 14, and can be altered by neuromuscular blockade, by administration of guanosine 3′,5′-monophosphate (cyclic GMP), and by varying the amount of target tissue in the developing chick embryo (*3, 6*).

Erichsen *et al.* (*7*) observed enkephalin-like immunoreactivity in presynaptic terminals in the ciliary ganglion before the onset of cell death. Cultures of ciliary ganglion cells, which lack preganglionic terminals, also show enkephalin-like immunoreactivity (*8*). These findings suggest that opiate peptides may exist both pre- and postganglionically in vivo. Since opiates modulate neurotransmission in many systems (*9*), we examined the possibility that opiates play some role in regulating normal cell death in the ciliary ganglion.

We administered morphine or naloxone or both daily to the vascularized chorioallantoic membrane through a window in the egg's shell on embryonic days 7 to 14. All experimental embryos survived; motility (*10*) was slightly less than in controls (9 versus 14 kicks per minute, respectively). The stages of the experimental and control embryos were determined with the criteria of Hamburger and Hamilton (*11*), including beak length, appearance of feather germs, third toe length, and eyelid morphology. The experimental animals were indistinguishable from the controls at all embryonic ages examined, suggesting that morphine does not have a general effect on growth and development. Some eggs were injected continuously until hatching; these chicks did not differ from the controls in their ability to hatch or their weight at hatching.

The embryos were killed on embryonic day 14 and the ciliary ganglia were fixed in Bouin's and embedded in paraffin. Serial sections (8 μm) were stained with hematoxylin and eosin orange and ganglion cells containing at least one nucleolus were counted at ×400 (Table 1). Daily treatment with 20 μg (*12*) of morphine sulfate (Mallinckrodt) resulted in only a moderate increase in cell survival; daily treatment with a larger dose (200 μg) had no significant effects. In contrast, daily injection of a progressively larger dose of morphine (20 to 200 μg) rescued most of the cells that would have died (total cell number being 5948 ± 345 on day 14), and this effect was reversed by naloxone. Daily treatment with 20 μg of naloxone alone did not significantly affect cell survival when administered during the period of normal cell death.

Table 1. Effect of morphine treatment on cell number (mean ± standard error) in the ciliary ganglion. All raw cell counts were corrected for overcounting (*24*). Values in parentheses give the number of experiments.

Treatment	Embryonic day			1 day after hatching
	8	14	16	
Control	6260 ± 339(9)	3704 ± 241(5)		3315 ± 116(8)
Morphine (20 μg/day)		4996 ± 584(3)*		
Morphine (200 μg/day)		4354 ± 275(3)		
Morphine† (20 to 200 μg/day)		5948 ± 345(3)‡		
Morphine† and naloxone (20 to 200 μg/day)		4199 ± 125(3)		
Morphine§ (20 to 200 μg/day)			4302 ± 509(3)	
Naloxone (20 μg/day)		4098 ± 307(3)		

*Significantly different from corresponding control value [$P < 0.05$, one-tailed Student's t-test with the Bonferroni inequality (*25*)]. †Eggs were injected daily with an increasing dose of morphine as follows: 20, 20, 40, 40, 100, 100, 200 and 200 μg/day. ‡$P < 0.025$. §Eggs were injected daily with the same increasing dose of morphine, followed by 2 days without injection.