A Multifunctional Cell Surface Developmental Stage-specific Antigen in the Cockroach Embryo: Involvement in Pathfinding by CNS Pioneer Axons

Lansheng Wang, Yun Feng, and Jeffrey L. Denburg
Department of Biology, University of Iowa, Iowa City, Iowa 52242

Abstract. mAb DSS-8 binds to a 164-kD developmental stage-specific cell surface antigen in the nervous system of the cockroach, Periplaneta americana. The antigen is localized to different subsets of cells at various stages of development. The spatial and temporal distributions of DSS-8 binding were determined and are consistent with this antigen playing multiple roles in the development of the nervous system. Direct identification of some of these functions was made by perturbation experiments in which pioneer axon growth occurs in embryos that are cultured in vitro in the presence of mAb DSS-8 or its Fab fragment. Under these conditions the pioneer axons of the median fiber tract grow but follow altered pathways. In a smaller percentage of the ganglia, the immunoreagents additionally produce defasciculation of a subset of DSS-8 labeled axons. Therefore, direct roles for the DSS-8 antigen in both the guidance of pioneer axons and selective fasciculation have been demonstrated.

One of the major goals of biochemical studies of development is an understanding of the role of cell surface molecules in the cell migrations, changes in cell shape and cell-cell adhesions that lead to morphogenesis. This is also the case in studies of the nervous system where there are a large number of neurons, each of which must extend an axon along a particular path and make synaptic contacts with specific targets. There are contrasting views as to the relative number of such cell surface molecules involved in these cellular events. One view suggests that there are relatively few such molecules that are widely distributed, involved in several cellular phenomena and whose spatial and temporal distributions change throughout development thus generating specificity (Edelman, 1984). An example of this type of molecule is the neuronal cell adhesion molecule (N-CAM) which eventually appears in nearly all neurons at some time during development as well as several locations outside the nervous system (reviewed in Cunningham et al., 1987). On the other hand, there are suggestions that there are a great number of different molecular labels with very restricted distributions that reflect the multiplicity of cell identity and position (Sperry, 1963). Examples of this type of molecule are the neuron subset specific antigens that are only found on the surfaces of axons of individual identified insect motor neurons (Kotrla and Goodman, 1984; Denburg et al., 1987).

However, in between these extreme cases of specificity of molecular localization there are molecules that at any moment of time are present on a very restricted subset of cells, but at various times are present on different subsets. In this communication we describe the distribution of one such molecule within the developing nervous system of the cockroach Periplaneta americana. It is initially identified by mAb DSS-8 that was previously selected for study because it transiently binds to several parts of the embryonic nervous system in comparison with its binding to that in the adult insect (Denburg et al., 1989). The restricted transient localization of this molecule to the apical surface of ectodermal epithelial cells that are undergoing any type of morphogenesis including invagination, evagination, and epiboly has previously been described (Norbeck and Denburg, 1990, 1991). The spatial and temporal distributions of the antigen within the nervous system correlate with different cellular events and suggest that the 164-kD protein is involved in multiple functions. Immunoperturbation experiments have directly demonstrated a role for the antigen in axon guidance and fasciculation.

Materials and Methods

Experimental Animals

Egg cases were collected daily from laboratory colonies of the cockroach, Periplaneta americana. The collected eggs were put in a plastic petri dish and stored in a 30°C humidified incubator with a 12:12 h light-dark cycle. The hatched nymphs were transferred to a new petri dish supplied with water and laboratory rat chow and also maintained under the same conditions.
Staging of Embryos

Determination of developmental stages of the embryos was based on the external morphology rather than the age of the embryo. This was done because morphological variation was observed among embryos from different egg cases collected on the same day. This variation was particularly significant at early stages where the external structures change rapidly within a short period of time. The system of Lenoir-Rousseaux and Lender (1970) was used because it provides detailed descriptions of morphology of early cockroach embryos. The percentage of development was calculated using 30 d as 100%. This is the time it takes nymphs to emerge from their egg cases under the incubating conditions mentioned above.

Production of the mAb

In the experiments involving the binding of mAb to tissue whole mounts, supernatants of cloned hybridoma cells are used. The DSS-8 clone was generated in a fusion using spleen cells from a mouse that had been immunized with nerve cords from 15 d (50% development) embryos (Denburg et al., 1989). For perturbation experiments, the mAbs were purified on a Protein A Affi-Gel column using procedures for mouse IgG recommended by the manufacturer (Bio-Rad Laboratories, Cambridge, MA). The monovalent Fab fragment was prepared by digesting the purified mAbs with papain using procedures described by Harlow and Lane (1988). Briefly, sodium acetate (pH 5.5) was added to the purified mAb to a final concentration of 100 mM; cysteine to a final concentration of 50 mM and EDTA 1 mM. Each milligram of mAb was incubated with 10 μg papain for 5 h at 37°C. The reaction was stopped by adding iodoacetamide to a final concentration of 75 mM and incubated at room temperature for 30 min. Any remaining intact antibodies and Fc fragments were removed by chromatography on a Protein A column before use. The purity of the final Fab fragments was checked by SDS-PAGE under nonreduced conditions in which the Fab fragments migrate as a 50-kd protein and the Fc fragments as a 25-kd one.

Protein concentrations of purified mAb and Fab were determined from the absorbance of UV light at 280 nm.

Binding of mAb to Whole Mounts of Embryonic Tissues and Nymphs

The detailed method of detecting mAb binding to the embryonic nerve cords in whole mounts was previously described (Denburg et al., 1989). Briefly, embryos were removed from the egg case and fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.2, for 30 min at room temperature. The specimens were rinsed in PBS and blocked in TBS (50 mM TBS, pH 7.2, 350 mM NaCl) to which was added 2% Triton X-100, 2% goat serum, 3% BSA for 1 h at room temperature. Then they were incubated in a mixture of hybridoma medium and blocking solution in a 1:4 ratio at 4°C for 12 h. Unbound antibody was removed by several washes with TBS containing 0.2% Triton X-100. The specimens were treated again with the TBS blocking solution for 30 min followed by a 12-h incubation at 4°C with goat anti-mouse secondary antibodies conjugated to HRP (HRP-GAM) (Bio-Rad Laboratories) diluted 1:200 with TBS blocking solution. Unbound secondary antibodies were removed by several washes with TBS containing 0.2% Triton X-100. The specimens were rinsed again with TBS and fixed in 4% paraformaldehyde for 2 h at room temperature. After two rinse washes with TBS, specimens were treated with the secondary antibody for 2 h at room temperature. After three rinses in PBS the presence of the antigen was detected with DAB and H2O2.

For thin plastic sections, the mAb was bound to the embryos using the previously described whole mount procedure. After staining with DAB the embryos or the nerve cords were dehydrated through a series of different concentrations of ethanol ranging from 50 to 100%. The specimens were then infiltrated and embedded in Polybed 812 (Polysciences, Inc., Warrington, PA). Sections of 1-μm thickness were cut using an Ultracut E Microtome (Reichert Jung, Vienna).

Staining of Neurons Using DiI

The fluorescent dye DiI (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) was used to label the axons and cell bodies of the neurons pioneering the anterior commissure and the dorsal median fiber tract. Its lipid solubility and diffusion within the plasma membrane enables it to be retrogradely and anterogradely transported from its site of injection (Schwartz and Agranoff, 1981; Hoonig and Humce, 1986, 1989). Examination with fluorescein microscopy (rhodamine filters) allows visualization of the complete axonal branching of any neurons near the injection site. The applicability of this technique to fixed tissue ensures the preservation of axonal structure (Godement et al., 1987). Embryos at the appropriate developmental stages were dissected in PBS and subsequently fixed in 4% paraformaldehyde. The specimens were transferred to a microscope slide for dye injection. DiI (Molecular Probes Inc., Junction City, OR) was solubilized in ethanol with a final concentration of 0.1%. Small volumes of the dye were injected from micropipettes with a Picospritzer II (General Valve Corporation). As a result of its poor solubility in aqueous solutions, the dye immediately precipitated at the site of injection and thus very localized applications within the small ganglia were possible. After injection the specimens were left in 4% paraformaldehyde at room temperature. They were periodically checked to monitor the labeling of axons.

Perturbation Experiments

Embryos were dissected in L-15 culture medium (Sigma Chemical Co., St. Louis, MO) supplemented with 4 mg/ml glucose, 150 μg/100-oxycocysteine (Sigma Chemical Co.), 8 μg/ml bovine insulin (Sigma Chemical Co.), 500 U/ml penicillin and 500 μg/ml streptomycin. Attempts were made to preserve the amniotic membrane and to remove all the yolk when possible. Embryos from the same egg case (typically there are 12-16 embryos in an egg case) were divided into two groups. One was cultured in the presence of purified DSS-8 or its Fab fragment and the other was cultured in the presence of a control immunoreagent. Both experimental and control immunoreagents were run in pairs that contained the same concentration of protein. The embryos were cultured in 400 μl of medium in a 24-well culture plate at 30°C for 60-72 h. Every 12 h, half the volume of medium was replaced with fresh medium containing the same concentration of the immunoreagent. At the end of the culture period, the embryos were rinsed in the medium to remove unbound antibodies and then fixed in 4% paraformaldehyde. When DSS-8 was the perturbant, its binding to the embryos was immediately detected with HRP-GAM as described in previous sections. When other control mAbs were the perturbants the axons of the anterior commissure and the median fiber tract were subsequently visualized in the cultured, fixed embryos by treating with DSS-8, and detecting the binding by the routine whole mount procedure.

Biochemical Characterization of the DSS-8 Antigen

Nerve cords from embryos at 83% development or whole embryos at 25% development are sonicated in PBS containing 2 mM PMSF, 50 mM EGTA, 17.5 μM leupeptin, and 5 μg/ml aprotinin. All further references to PBS will include these inhibitors of proteolytic enzymes. The final protein concentration is 2-3 mg/ml. The sonicate is centrifuged at 100,000 g for 1 h and the resulting PBS pellet is resuspended in various solutions in attempts to extract the antigen. After recentrifugation of these suspensions, the supernatants are saved, and the pellets are resuspended in PBS. Semi-quantitative measurements of the amounts of antigen in these fractions are performed using the immunodot blot procedures of Hawkes et al. (1982).
In outline, the immunoprecipitation and fractionation of the DSS-8 antigen entails the biotinylation of all proteins, solubilization in 1% CHAPS, incubation with immobilized mAb, extensive washing of the antigen–mAb complex, elution of the antigen, fractionation by SDS-PAGE, transfer to nitrocellulose, and detection with alkaline phosphatase–streptavidin. In a typical experiment 150 nerve cords (200–300 μg protein) are sonicated in 150 μl PBS. The protein is biotinylated by the addition of 3 μl of N-hydroxysuccinimide biotin (10 mg/ml DMSO) and incubation for 4 h at room temperature with continuous shaking. The reaction is stopped by the addition for 2.4 μl NH4Cl (1 M) and centrifugation at 100,000 g for 0.5 h. The pellet is washed twice in PBS by resuspension and recentrifugation. The antigen is then solubilized by overnight extraction at 4°C with 1% CHAPS in PBS followed by centrifugation at 100,000 g for 1 h. mAb DSS-8 is immobilized by incubating 0.5 ml of hybridoma supernatant for 2 h at room temperature with rabbit anti-mouse serum that is covalently attached to polyacrylamide beads (Bio-Rad Laboratories). The beads are washed twice with 500 μl of PBS in order to remove unattached mAb. A control neuron-specific mAb that binds to the chick spinal cord but not to any cockroach tissue is immobilized in an identical manner. The immobilized control mAb is then added to the detergent extract and incubated with shaking for 1.5 h at room temperature. After a low speed centrifugation the supernatant is incubated in an identical manner with immobilized DSS-8 and the eluate is discarded. Both sets of immunobeads are washed three times with 1 ml of 1 M NaCl in PBS and another three times with PBS. Antigen bound to the beads is eluted in 60 μl of sample buffer containing 1% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.62 M Tris, pH 8.8. After heating at 90°C for 5 min the samples are fractionated by SDS-PAGE in a 7.5% acrylamide gel (Laemmli, 1970) and then transferred to nitrocellulose (Burnette, 1981). The blots are blocked for 1 h in 0.05% Tween 20, 1% powdered milk in TBS, and then incubated for 1 h in 1/3,000 dilution of alkaline phosphatase–conjugated streptavidin in the blocking solution. After washing three times with PBS the immunospecific proteins are made visible by treatment with the chromogenic substrates NBT (330 μg/ml) and BCIP (165 μg/ml) in 0.1 M Tris, pH 9.5, 150 mM NaCl, 4 mM MgCl2.

Results

The central nervous system of the cockroach embryo consists of a ventral nerve cord composed of a brain and a chain of segmental ganglia. In each ganglion, axon tracts are arranged in an orthogonal scaffold consisting of two longitudinal connectives and two commissures. The connectives link the adjacent ganglia along the anterior–posterior axis of the nerve cord. The anterior commissure and posterior commissure extend across the midline of the ganglion and contain several different axon fascicles. In addition, there is a dorsal median fiber tract that extends along the midline of the ganglion.

mAb DSS-8 was originally selected for study because it labels a subset of axons in the anterior and posterior commissures in ganglia from embryos at stage 25 (50% development) and it does not label these structures in the adult ganglia (Denburg et al., 1989). As a first step in the determination of the function of the DSS-8 antigen, a complete developmental time course of the binding of mAb DSS-8 to the central nervous system (CNS) has been performed. It shows that in addition to the above mentioned cellular structures, mAb DSS-8 transiently labels the ventral midline neuroepithelial cells, the median neuroblast, and some of its progeny, the pioneer axons of the anterior commissure, the pioneer axons of the median fiber tract, extracellular matrix along the midline, and the neuropile. These results will be described in detail. Because there is a developmental gradient along the anterior–posterior axis of the nerve cord, and in order to be consistent, all of the figures represent views of the mesothoracic region or ganglion (T2) unless otherwise specified. All observations are based on examinations of at least 30 specimens.

Binding of DSS-8 to the Pioneer Axons of the Anterior Commissure

The binding of DSS-8 to axons in the anterior commissure is observed at progressively early stages of development. The first binding is detected at stage 14 (19% development) to two groups of cells in each hemiganglion at the level of the future anterior commissure and slightly lateral to the longitudinal connectives. These groups of cells are very close to each other and the more anterior one is also more ventral (Fig. 2 a, arrows) compared to the slightly more lateral one (Fig. 2 a, open arrows). The labeling of about four to six cells in each group by DSS-8 is very light. One of the cells in each anterior group extends an axon that grows toward the midline, crosses it, and fasciculates with the homologous axon from the opposite side (Fig. 2, b and c). These axons remain tightly fasciculated and continue to grow anteriorly to join the contralateral longitudinal connectives. The final destination of the axons is not clear. The binding of DSS-8
to the two axons is not distributed homogeneously on their surfaces. When the axons first start to grow and before they meet each other at the midline, the binding is light. As soon as they fasciculate with each other the mAb binding becomes stronger (Fig. 2 c, arrow). In addition, the levels of DSS-8 binding are always higher in the parts of the axon in the anterior commissure than in those in the longitudinal connectives. It is also observed that before these first two axons encounter each other there is a low level of DSS-8 binding in the region of the midline towards which the axons are growing (Fig. 2 b, open arrow). This binding is not closely associated with the plasma membrane or cytoplasm of any of the surrounding cells suggesting an extracellular localization. This labeling is of relatively low intensity and may possibly be interpreted as non-specific background. However, other mAbs, like that in Fig. 2 d do not show such binding at this site. By varying the plane of focus, it can also be shown that this binding is not due to DSS-8 labeling of the out of focus midline neuroepithelial cells.

It appears as though these first two DSS-8-labeled axons are pioneering the anterior commissure. Additional evidence for this comes from three observations. First, microscopic examination with Nomarski contrast optics of many DSS-8 labeled and freshly fixed and unlabeled specimens shows that at stage 14 (19% development) the DSS-8 positive axons are the only axons present in the anterior commissure (data not shown). Second, mAb 16-7G12 which binds to a cytoplasmic component present in all axons (Denburg et al., 1986), labels only two axons in the region of the anterior commissure at this stage of development (Fig. 2 d). Third, when DiI is injected into the longitudinal connectives on each side of the stage 14 ganglion at a level slightly posterior to the anterior commissure, all axon pathways present at this stage fluoresce. These include the anterior commissure with two axons connected to cell bodies in the region previously described to contain DSS-8-labeled neurons (Fig. 2 e and f). In addition, the progeny of the midline precursor cells (MP1 and MP2) whose axons pioneer the longitudinal connectives (Bate and Grunewald, 1981) become fluorescent (Fig. 2 e). Attempts to detect DSS-8-labeled pioneer axons using immunoelectron microscopy have not been successful. However, it is apparent that at stage 14 (19% development) when
Figure 2. Binding of DSS-8 to pioneer axons in the anterior commissure. (a) Two groups of cells are lightly labeled by DSS-8 at stage 14 (19% development) before the formation of the anterior commissure. One cell in each anterior group (black arrows) will extend an axon toward the midline to pioneer the anterior commissure. The posterior group of DSS-8 labeled cells is indicated with open arrows. (b) At late stage 14 (19% development) the two DSS-8-labeled axons are seen in the process of forming the commissure (black arrows). A low level extracellular expression of the DSS-8 antigen is observed on the midline before the two pioneering axons meet at the midline (open arrow). (c) At early stage 15 (20% development) the expression of the DSS-8 antigen is stronger after the two axons cross the midline and fasciculate with their counterpart (open arrow). (d) A sibling embryo of the one in B is treated with mAb 167G12 which labels all axons present and only two axons (black arrows) are detected in the commissure. In addition the midline precursors (open arrows) whose axons pioneer the longitudinal pathways are labeled. (e) An embryo at the same developmental stage as that in C is injected with DiI and the first axons in the commissure (black arrow) fluoresce, as does the out of focus neuronal cell body connected to one of these axons (arrow head). The midline precursors are also labeled (open arrows). (f) The same specimen is focused slightly ventrally to show the two cells whose axons pioneer the anterior commissure. Anterior is to the top. Bar, 20 μm.

DSS-8 labels only two axons in the anterior commissure, that only two axons are detected in this region using three other techniques.

**Binding of DSS-8 to Pioneer Axons of the Dorsal Median Fiber Tract**

The dorsal median fiber tract has been shown in grasshoppers to be pioneered by axons of the two progeny of midline precursor 4 cell (MP4) (Goodman et al., 1981). It is also the pathway taken later by the axons of some of the progeny of the median neuroblast. The first binding of DSS-8 in the vicinity of the median fiber tract is observed at stage 15 (20% development). There is no labeling of any axons in the tract nor of the MP4 progeny. Instead, the path along which the axons will grow is lightly labeled (Fig. 3 a). This binding is not clearly associated with any of the surrounding cells and appears extracellular in nature. Another ganglion at the same stage of development treated with mAb 16-7G12 shows that there are no axons yet in the median fiber tract and no low-level non-specific binding in the region where the tract will form (Fig. 3 b). Axons in the median fiber tract are first labeled with DSS-8 at early stage 16 (23% development) (Fig. 3 c). The cell bodies of the two progeny of the MP4 are labeled as well as their axons which extend anteriorly along the midline between the two MP3 cells. Each of the axons bifurcates just before the anterior commissure and grows laterally along this pathway during stage 17 (25% development) (Fig. 3 d). Evidence that these DSS-8-labeled axons pioneer the median fiber tract includes the failure to detect any other axons in this pathway at an earlier stage of development using microscopy with Nomarski optics or in ganglia treated with the neuron-specific mAb 16-7G12. In addition, the injection of DiI on the midline of the anterior half of the ganglion during stage 16 (23% development) retrogradely labels the median fiber tract and the two progeny of the MP4.
Figure 3. Binding of DSS-8 to pioneer axons in the median fiber tract. (a) DSS-8 first labels extracellular matrix along the midline in stage 15 (20% development) embryos. This labeling of the path of the median fiber tract occurs before there is any pioneer axon growth. (b) A ganglion from an embryo at the same stage of development treated with the neuron-specific mAb 16-7G12 does not label the extracellular matrix along the midline. No axons have formed yet in this tract. (c) At stage 16 (23% development), the two MP4s (open arrow) are observed to extend the axons to pioneer the median fiber tract (arrow). The tips of the growth cones have just arrived at the level of the anterior commissure and are about to bifurcate (arrow head). (d) At stage 17 (25% development) these axon have bifurcated (open arrow) and have grown laterally along the anterior commissure. (e) Retrograde labeling with DiI of a stage 16 (23% development) embryo detects the median fiber tract (arrow) and in f the cell bodies of the two MP4s (arrows) that pioneer the tract. Anterior is towards the top. Bar, 20 μm.

(Fig. 3 e and f). No other neuronal cell bodies with axons in this tract are detected.

**Binding of DSS-8 to the Neuropile Region**

In stage 18 (27% development) embryos mAb DSS-8 starts labeling two areas along the midline in addition to the subset of axons. Transverse sections through these structures show that they are neuropile—regions of high density of synapses among neuronal processes (Fig. 4 a). At stage 26 (53% development) a larger and more widespread neuropile becomes labeled by DSS-8. This occupies almost the entire central region of the ganglion and begins to obscure the previously DSS-8 positive fascicles (Fig. 4 b). By stage 27 (66% development) DSS-8 binding to this region becomes so intense that the subset of DSS-8-labeled pathways is almost unobservable in the whole mount preparations (Fig. 4 c). The binding of DSS-8 to the neuropile is maximal just before the embryos hatch (Fig. 4 d) and in sections of labeled ganglia at this stage the binding is observed throughout the neuropile (Fig. 4 e). This pattern of DSS-8 binding continues into the nymphal development. By 46 d after emergence from the egg case DSS-8 binding to the neuropile of the nymphal ganglion becomes less intense in the central region (Fig. 4 f). The binding of DSS-8 to the neuropile further decreases as seen in a ganglion from a 76-d-old nymph (Fig. 4 g). By 117 d after emergence the amount of the DSS-8 antigen in the neuropile (Fig. 4 h) is identical to that in the adult ganglion (see Fig. 3 B in Denburg et al., 1989).

**Perturbation of Neurodevelopment in Embryos Cultured in the Presence of DSS-8**

Of all the sites of DSS-8 binding in the developing nervous system, those on the pioneer axons of the anterior commissure and the medial fiber tract are the most amenable for observations of perturbations produced by exogenous immunoreagents. mAb DSS-8 or its monovalent Fab fragment can be added to medium in which embryos are cultured for an interval of time sufficient for these axons to grow. For such perturbation experiments to be successful it must first be demonstrated that the axons pioneering these tracts grow along the normal path in the cultured embryos and that these growing axons are accessible to the immunoperturbant. Both of these requirements have been satisfied.
The axons that pioneer the first fascicle in the anterior commissure start growing at stage 14 (19% development) and have completed the tract by stage 15 (20% development). In perturbation studies of this axon tract, embryos at stage 13 (18% development) in which axon growth in this pathway has not yet started, are cultured for 72 h. In vivo this time interval will have brought the embryo to 28% development. However, the cultured embryos develop at about half the normal rate, and are thus at a morphological stage 16 corresponding to 23% development. In all (n = 225) embryos cultured with no or control immunoreagents the anterior commissure forms (Fig. 5a) in a manner indistinguishable from that formed in vivo (Fig. 2c). The axons that pioneer the median fiber tract start growing at late stage 15 (20% development) and have completed the pathway by stage 17 (25% development). In perturbation studies of this axon tract embryos at stage 14 (19% development) in which axon growth has not yet occurred, are cultured for 72 h. After this interval the embryos in vivo would be at 29% development, but after being in culture are observed to be at morphological

Figure 4. Binding of DSS-8 to central neuropile. (a) In a frozen transverse section through the anterior commissures of a stage 18 (27% development) embryo, DSS-8 labels a restricted neuropile region in the middle and three commissural pathways connected to it. Two groups of ventrally located cell bodies are also labeled. (b) At stage 26 (53% development) a larger general neuropile region starts to express the DSS-8 antigen. This becomes obvious at stage 27 (66% development) (c) and the binding of DSS-8 to the neuropile reaches its maximum just before hatching, 100% development (d). (e) A plastic transverse section of a stage 30 (100% development) ganglion shows the binding of DSS-8 to the general neuropile region (ventral is to the top). (f) Binding of DSS-8 starts to decrease in a ganglion from a 46-d nymph. (g) In the ganglion from a 76-d nymph, the pattern of DSS-8 binding starts to resemble that of the adult. (h) The pattern of binding to a ganglion from a 117-d-old nymph is identical to that of the adult. Bar, 20 μm.
Figure 5. The growth of the axons pioneering the anterior commissure and the median fiber tract in cultured embryos and their labeling with exogenous mAb DSS-8. In embryos cultured for 72-h the pioneer axons of the anterior commissure (a, arrows) and of the median fiber tract (b, arrow) grow along a path identical to that followed in vivo. These axons are made visible by labeling with DSS-8 after the culture period. When the mAb is present during the culture period, the same axons become labeled (c and d) indicating that the surface antigen is accessible to the exogenous immunoperturbant. These samples are among those not perturbed by the mAb. Bar, 20 μm.

stage 17 or 18 corresponding to 25–27% development. These embryos develop in vitro at a rate ~70% of that in vivo. In 93% (n = 675) of the ganglia from embryos cultured with no or control immunoreagents the median fiber tract forms (Fig. 5 b) in a manner indistinguishable from that formed in vivo (Fig. 3 d). In these experiments the axon tracts are visualized by labeling with DSS-8 after the culture period.

To demonstrate that the surface antigen is accessible to immunoperturbants, living embryos are cultured in the presence of mAb DSS-8. After extensive washing to remove unbound mAb the embryos are fixed and treated with secondary antibodies. In those ganglia in which no perturbation is observed, the same axons in the anterior commissure (Fig. 5 c) and the median fiber tract (Fig. 5 d) are labeled as in fixed, permeabilized preparations. This indicates that in embryos cultured in the presence of immunoperturbants the DSS-8 antigen on the surface of the axons forms a complex with the antibodies.

Control embryos are incubated in the presence of mAb DSS-3 or its Fab fragment which binds to a cell surface antigen on all embryonic neurons (Denburg et al., 1989). Previous studies have shown that DSS-3 binds to living, intact embryos at the stages examined here (Denburg and Norbeck, 1989). These controls eliminate the effects of having an immunoreagent of the same isotype, IgG1, in the tissue culture medium and binding to a cell surface antigen other than DSS-8. After culturing in the presence of mAb DSS-3, the embryos are fixed and labeled with DSS-8 for the visualization of the anterior commissure and the median fiber tract. In spite of the higher background binding caused by DSS-3, the pioneer axons of the anterior commissure and the median fiber tract can always be detected after labeling with DSS-8.

Embryos are cultured in the presence of various concentrations of immunoperturbants. This study is based on observations of a total of 740 experimental ganglia cultured in the presence of mAb or Fab DSS-8 and of 675 control ganglia cultured in the presence of DSS-3 immunoreagents or with no reagents added. Although there are three thoracic and 10 abdominal ganglia in an embryo, on the average only three or four of them are analyzed. This occurs because during the culture period there is insufficient axon growth in several of the ganglia as a result of the developmental gradient that exists along the anterior–posterior axis of the embryo. In addition, during mounting of the preparations all ganglia are not appropriately aligned for suitable microscopic analysis. Embryos are rarely observed that have all or no ganglia perturbed. This indicates that there is not a subpopulation of embryos that is particularly susceptible to perturbation.

The major criterion for perturbations of the median fiber tract pioneer axons is an alteration in the path followed by the growing axons. They no longer grow along the midline between the two large MP3 cells, but instead extend anteriorly along a more lateral path (Fig. 6). Such perturbations are observed in 254 (34%) of the total experimental ganglia. By comparison, only 47 (7%) of the total control ganglia exhibited a similar alteration in axon growth. Therefore, the incidence of errors in the path of median fiber tract pioneer axons is greatly increased by the addition of mAb or Fab DSS-8. These perturbation studies have concentrated on the pioneer axons of the median fiber tract. To demonstrate that this perturbation of the pioneer path results in the misdirection of the subsequent axon tract, it is necessary that a significant amount of secondary axon growth occurs. This does not happen in the 72-h culture period used and embryos are not healthy after longer times in vitro.

The perturbed median fiber tract pioneer axons are observed to follow three different alternate paths. If the axons remain fasciculated they extend along the lateral surface of either the left (Fig. 6 b) or the right MP3 cell (Fig. 6 c).
Figure 6. The immunoperturbation of the median fiber tract in ganglia from embryos cultured in vitro. The pathway is visualized with mAb DSS-8. In the presence of Fab DSS-8 as a perturbant the pioneer axons of this tract (arrows) defasciculate (a) in some ganglia and each follows a more lateral path until reaching the anterior commissure. In many ganglia the pioneer axons of the median fiber tract remain fasciculated but take either a path to the left (b) or to the right (c) of the midline (arrowhead). Anterior is toward the top. Bar, 20 μm.

There is no preference for growth to either side. In several ganglia the two median fiber tract pioneer axons defasciculate. In all cases where defasciculation occurs one of the axons extends along the lateral surface of the left MP3 cell and the other along that of the right cell (Fig. 6 a). Defasciculation is observed in 68 (9%) of the total 740 experimental ganglia and in 27 (4%) of the total 675 control ganglia. Therefore, although defasciculation occurs in only 27% of all the ganglia with perturbed axon growth, this represents a doubling of the incidence by the DSS-8 immunoperturbers in comparison to controls. In summary, in all perturbations of the median fiber tract pioneer neurons there is an alteration in the path the axons follow although they always still grow anteriorly and bifurcate when they reach the anterior commissure. This is a robust phenomenon observed in a relatively high percentage of ganglia. This indicates a role for the DSS-8 antigen in axon guidance. The evidence for a role in axon fasciculation is less robust but still significant.

The previously discussed incidences of perturbations were calculated on the basis of the total set of experimental ganglia. However, the frequency with which these perturbations occur is observed to depend on the nature of and the concentration of the immunoreagents. To better compare the relative effectiveness of intact mAbs with their Fab fragments the amounts of these immunoreagents are represented in terms of the micrometer concentration of antigen binding sites (100 μg/ml of 150-kDa bivalent mAb is 1.33 μM antigen binding sites; 100 μg/ml of 50-kDa monovalent Fab is 2.0 μM antigen binding site). With mAb DSS-8 as the reagent perturbations are observed maximally in 29% of the ganglia (n = 73) with 12% of them being defasciculated. In comparison, when Fab of DSS-8 is the reagent, perturbation is observed maximally in 55% of the ganglia (n = 74) with 22% of them being defasciculated. To further investigate the relative effectiveness of perturbation by mAb DSS-8 and its Fab fragment, similar experiments are done at several concentrations of immunoreagents within the range of 18 nM and 2 μM antigen binding sites. The results are presented graphically in Fig. 7 along with those using mAb DSS-3 and its Fab fragment as controls. Each point corresponds to observations of between 25 and 98 ganglia from cultured embryos. Other controls using no immunoreagents are not significantly different from those using DSS-3. It is observed that the maximum frequency of total perturbations is produced at 0.16 μM antigen binding sites for both the mAb and the Fab of DSS-8 (Fig. 7 a). Further increases in concentration of immunoreagent up to 2.0 μM antigen binding sites does not further increase the frequency of perturbations. Similar results are observed for the perturbations producing defasciculation (Fig. 7 b). Here too the monovalent Fab DSS-8 is a more potent perturbant than the intact mAb. In fact, it is only at one concentration that mAb DSS-8 produces defasciculation at a significant level in comparison to control mAb DSS-3. The apparent decrease in perturbations at higher concentrations of immunoreagents is not significant. Therefore, in the dose–response curves the maximum incidences of perturbations are observed to plateau at levels <100%. In addition, for both defasciculation and alteration in the path of median fiber tract axons, the Fab DSS-8 produces a higher incidence of perturbations than the mAb DSS-8. This occurs in spite of the observation that the Fab does not bind to additional sites in the embryo and does not label with greater intensity previously detected sites.

In contrast to these studies demonstrating perturbation of the median fiber tract, all attempts to perturb the formation of the anterior commissure were unsuccessful. Concentrations of mAb and Fab DSS-8 as high as 2.0 μM were used, and labeling of the axons with immunoreagents was observed during the culture period. Therefore, these two pathways in which the DSS-8 antigen is present in the pioneer axons, differ greatly in their susceptibility to perturbation by the DSS-8 immunoreagents.
Figure 7. Quantitative analysis of the perturbations in the median fiber tract in ganglia from cultured embryos resulting from the presence of DSS-8 immunoreagents in the culture medium during the formation of this tract. (a) Graphic representation of the incidence of perturbations, altered paths of growth, as a function of the concentration of antigen binding sites. (b) Similar graph showing the incidence of defasciculation as a function of antigen binding sites. The perturbants are mAb DSS-8 (●——●), Fab DSS-8 (○—○), and controls mAb DSS-3 (▲—▲), Fab DSS-3 (▼—▼).

Biochemical Characterization of the DSS-8 Antigen

Maximum levels of the DSS-8 antigen are immunohistochemically detected in the embryonic nervous system between stage 28 (83% development) and hatching. Nerve cords from embryos at these stages are initially used as a source of antigen. After solubilization of the tissue in SDS (concentrations between 0.1-1.0%) and fractionation by SDS-PAGE, no antigen is detected in Western blots. The absence of boiling or of the reducing agent, β-mercaptoethanol failed to prevent the loss of DSS-8 binding to the antigen. Varying the composition of the blocking solution also failed to enable the detection of the antigen. Therefore, immunodotblot assays are used to semiquantitatively determine the levels of the antigen in various solutions used to attempt to solubilize the antigen in a form that will still be recognized by the mAb. DSS-8 antigen remains insoluble after extraction with 1 M KCl, 0.001 M Tris buffer, or 0.1 M Na₂CO₃, pH 11.0. Treatment with non-ionic detergents (1% Triton X-100 or Tween 20) destroys antigenicity in both supernatants and pellets. However, 1% octyl glucoside or CHAPS solubilizes a specific mAb binding component. When CHAPS solubilized membranes are treated with DSS-8 a 164-kD protein is specifically immunoprecipitated (Fig. 8). In embryos at stage 17 (25% development) DSS-8 binds to only a small subset of axons in the ganglia (Fig. 3 d) and a subset of ectodermal epithelial cells about to undergo morphogenesis (Norbeck and Denburg, 1990, 1991). When membranes from whole embryos at this stage of development are solubilized in CHAPS and treated with DSS-8 the same 164-kD protein is specifically immunoprecipitated (Fig. 8). This indicates that DSS-8 binds to a single protein antigen at various of development both inside the nervous system and in the ectodermal epithelium.

Discussion

mAbs that transiently label parts of the embryonic nervous system at 50% development had previously been produced and saved for study because of the assumed likelihood that antigens with such a temporal distribution would play a role in the development of the nervous system (Denburg et al., 1989). The detailed description of the pattern of binding of one of these developmental stage specific mAbs (DSS-8) is presented in this communication. Binding of this mAb is observed to appear and disappear at different locations and at different times. The immunoprecipitation of a single 164-kD from embryos at various ages suggests that a single antigen is involved and that it is playing multiple functions. Some clues as to the identification of these functions may be inferred from the spatial and temporal distributions of the antigen during embryonic development.
The DSS-8 Antigen Is a Marker for Subsets of Various Types of Cells

Within the developing nervous system mAb DSS-8 transiently labels subsets of three types of cells. These include: (a) The midline epithelium, a subset of ventral epithelial cells all of which have the potential to become CNS neurons or glia; (b) the median neuroblast and its progeny, a subset of all neuroblasts from which the CNS is produced; (c) pioneer axons of the anterior commissure and the median fiber tract as well as secondary axons in these tracts and the posterior commissure, subsets of all axons within the CNS.

The midline neuroepithelium is a strip of cells which in Drosophila all delaminate dorsally from the ventral surface to form mitotic precursors of neurons and glia (Poulson, 1950; Thomas et al., 1988; Jacobs and Goodman, 1989) and which have previously been distinguished from neighboring cells on the basis of their morphology (Poulson, 1950) and their content of nuclear proteins (Crews et al., 1988). The binding of DSS-8 to these cells in the cockroach shows that they also have distinctive cell surface epitopes. The DSS-8 antigen is primarily localized to the apical surface of this group of cells. The midline neuroepithelial cells are all involved in the same developmental processes, the generation of a subset of neuronal precursors and the formation of commissural and medial axon tracts (Klambt et al., 1991).

The median neuroblast arises by dorsal delamination from the DSS-8-labeled midline neuroepithelial cells. However, unlike these cells its surface is labeled by the mAb around its entire circumference. The progeny of this neuroblast are labeled in a similar manner. The DSS-8 labeling of these cells is not just a reflection of their origin from the midline neuroepithelium; other cells that are derived from the midline neuroepithelium, like the MPI cells, are not labeled by DSS-8. The median neuroblast and its progeny remain aggregated together for a considerable period of development. This aggregate, all of whose cells are labeled with DSS-8, is structurally distinct from surrounding cells. All of the neuronal progeny of the median neuroblast, which will become the dorsal unpaired median cells, have similar properties in that they initially extend axons along the median fiber tract previously pioneered by the MP4 cells (Goodman and Spitzer, 1979) and that they use octopamine as a neurotransmitter (Hoyle, 1975; Dyndom and Evans, 1979).

DSS-8 binds to subsets of axons in different regions of the ganglia. Within each region the labeled axons fasciculate with each other to form major tracts. These include the anterior commissure, the posterior commissure, and the median fiber tract. The antigen is present at the time axons are being added to these tracts and could indicate a role in selective fasciculation. This is supported by the unusual distribution of the antigen within the axon branches of the neurons. The intensity of DSS-8 labeling is higher in segments of the axons within the commissures than in segments of the same, still fasciculated axon extending in the connectives. Similarly, when these axons are initially growing towards each other they are labeled less intensely than after fasciculation has occurred. In many of these characteristics DSS-8 is very similar to mAbs that have been previously used to identify fasciculins that have been suggested to play a role in selective axon fasciculation in the nervous system of other insects (Bastiani et al., 1987; Patel et al., 1987; Carr and Taghert, 1988; Harrelson and Goodman, 1988; Bieber et al., 1989; Snow et al., 1989; Grenningloh et al., 1991).

For some of the axon tracts labeled with DSS-8 it has been demonstrated that the axons that pioneer the pathway also possess the antigen. This might just reflect the fact that there are two axons pioneering the path that are fasciculated to each other throughout their growth as in the pioneers of the median fiber tract. However, this is not likely to be the case with the pioneers of the anterior commissure. These axons are labeled with DSS-8 before fasciculation. This early presence of the antigen might be in anticipation of the fasciculation that will soon occur. However, it is also possible that the DSS-8 antigen present on subsets of pioneer axons is additionally playing a role in the ability of these axons to respond to environmental cues that direct the path of growth.

Each of these subpopulations of cells labeled with DSS-8 perform a function that is distinct from that of unlabeled neighboring cells. This situation is similar to that observed in the ectodermal epithelium, where cells that will undergo any of three types of morphogenesis (invagination, evagination, and epiboly) are labeled with DSS-8 while neighboring unlabeled cells undergo no change in shape (Norbeck and Denburg, 1990, 1991). Perhaps the DSS-8 antigen helps to identify a group of cells that are in close communication with each other and that will respond as a unit to external signals.

Other Locations of the DSS-8 Antigen Suggest Additional Functions

Other sites of DSS-8 binding do not label subsets of functionally related cells. These include the CNS neuropile and the extracellular matrix. The highest levels of DSS-8 binding in the CNS are found during the second half of embryonic development and in the beginning of nymphal development. During this interval DSS-8 labels the entire neuropile. This suggests that at this time the antigen is involved in a process that occurs in most if not all neurons, not just the small subset of neurons that are labeled earlier in development. During this period of development there is continuous branching of motor neuron dendrites, sensory axon terminals, and interneuron axons and dendrites in the DSS-8-labeled regions. In this CNS neuropile, synapse formation, and maturation among these neurons is occurring and the DSS-8 antigen may be involved in some of these processes.

Of all the locations of DSS-8 binding, the extracellular matrix along the paths of growth of some pioneer axons is the one most difficult to detect (Figs. 2 b and 3 a). The intensity of mAb labeling is relatively low and it is not associated with any cells. However, this type of labeling is clearly not present when binding of other mAbs is examined (Figs. 2 d and 3 b). In addition, careful examination clearly shows that this pattern is not produced by underlying labeled cells that are out of the plane of focus. This extracellular labeling is present before there is axon growth and is primarily localized along the midline. It is particularly apparent in the region of the anterior commissure. These observations lead to the suggestion that the DSS-8 antigen is an environmental cue that directs the growth of the anterior commissure pioneer axons towards the midline and that keeps the anteriorly growing median fiber tract pioneer axons in a path along the midline.

Of these multiple hypothetical functions for the DSS-8 antigen direct evidence is presented demonstrating a role in...
guidance of pioneer axons and suggesting a role in axon fasciculation.

**DSS-8 Perturbation of Axon Fasciculation**

Selective fasciculation has been proposed to play an important role in the guidance of axon growth and arises from the recognition of an axon surface molecule by another axon usually possessing the same molecule (Goodman et al., 1982). Experimental evidence for the existence of this cellular phenomenon has been obtained (Raper et al., 1983a, b, 1984; Bastiani et al., 1984; du Lac et al., 1986). Molecules that have been demonstrated to play a role in selective fasciculation of subsets of axons in insect nervous systems include: fasciclin I (Jay and Keshishian, 1990) and fasciclin II (Harrelson and Goodman, 1988; Grenningloh et al., 1991).

Perturbation experiments were done with mAb DSS-8 or its Fab fragment in embryos cultured in vitro in order to determine if its antigen plays a role in axon fasciculation. Of all the axon tracts labeled by DSS-8 only the pioneer axons of the median fiber tract are defasciculated by this immunoperturbation. This specificity does not result from a differential accessibility of the immunoreagents to the antigens on the various axon tracts because when embryos are cultured in the presence of the DSS-8 immunoreagents the same subsets of axons are labeled as when the embryos are fixed and permeabilized in detergent before treatment with antibody. In addition, the intensity of labeling is the same in cultured embryos as in the fixed, permeabilized ones. Therefore, it would appear that while multiple molecular interactions are involved in axon fasciculation, the contribution of the DSS-8 antigen to this phenomenon in the median fiber tract pioneers is greater than in other axon tracts. However, this defasciculation is not a robust phenomenon.

The maximum incidence of defasciculation is 22% (n = 74) in ganglia from embryos cultured in the presence of 0.16 μM Fab DSS-8. At this same concentration of control Fab DSS-3 defasciculation is observed in only 1% (n = 66) of the ganglia. However, there is considerable variation in the low levels of perturbations at other concentrations of immunoreagents. Therefore, when the incidences of defasciculation within the total number of experimental (9%, n = 740) and control (4%, n = 675) ganglia are considered, the results do not appear as significant (Fig. 7b). Increasing the concentration of the Fab fragment does not increase the incidence of this perturbation. The failure of the immunoreagents to induce defasciculation in a higher percentage of the ganglia most likely occurs because the Fab DSS-8 only partially inhibits the function of the antigen or because multiple adhesion and recognition molecules are involved (Harrelson and Goodman, 1988).

It is possible to demonstrate that DSS-8 immunoreagents can specifically perturb the path of axon growth independent of their effects on axon fasciculation. For the pioneer axons of the median fiber tract defasciculation will almost by necessity cause at least one of the two axons to follow a different path. However, it is observed that whenever defasciculation is produced by a DSS-8 immunoreagent both axons follow a different path anteriorly towards the anterior commissure. Neither of them grow along the midline. In addition, alterations in the path of axon growth are observed maximally in 55% of the ganglia in embryos cultured in the presence of Fab DSS-8. This is a much higher incidence of perturbation than that maximally observed for defasciculation (22% of the ganglia). In most of the perturbed ganglia the pioneer axons
remain fasciculated but grow along a different path—either to the left or the right of the MP3 cells. Therefore, the same DSS-8 antigen may be independently involved in selective fasciculation and the ability to respond to environmental guidance cues.

The path of growth of the median fiber tract pioneer axon in the presence of DSS-8 immunoreagents indicates the involvement of multiple guidance cues. In all ganglia where their path of growth is perturbed, the pioneer axons still extend anteriorly until they approach the anterior commissure where they bifurcate and branch laterally in both directions. This means that at least three sets of guidance cues and the molecules needed to detect and respond to them are still functioning normally in neurons growing axons in the presence of DSS-8 immunoreagents. These cues control the anterior growth, the bifurcation at the anterior commissure and the lateral growth along the anterior commissure of the axons. The DSS-8 immunoreagents selectively perturb axon growth along the midline. The extracellular localization of the DSS-8 antigen along the midline is consistent with it being the cue required for axon growth along this path.

Further evidence for this comes from the observation that monoclonal Fab DSS-8 is a more effective perturbant of the path of axon growth than is the bivalent mAb. Perturbations in the path of axon growth are observed maximally in 55% of the ganglia cultured with Fab DSS-8 and in only 28% of the ganglia cultured with mAb (Fig. 7a). Increasing the concentration of immunoreagent another 10-fold does not increase the incidence of perturbation. Again, the explanation for these observations in terms of decreased accessibility of the mAb to the antigens is not relevant. No difference in the intensity of labeling of the median fiber tract pioneer axons and the midline extracellular matrix is observed with the Fab and the mAb. Increasing the concentration of immunoreagents above that producing maximum percentage of perturbations does not increase the intensity of labeling of these sites. It is proposed that the decreased effectiveness of the bivalent mAb arises from its ability to cross-link extracellular DSS-8 with forms of the antigen on the axon surface. Again, it cannot be determined whether the axon surface form of the DSS-8 antigen is the normal receptor for the extracellular matrix form that is acting as a guidance cue. It is possible that the artificial cross-linking of these two forms of the antigen just mimics the normal ligand–receptor interaction.

Comparison of DSS-8 Antigen with Other Neuronal Molecules

Although only one 164-kD form of the DSS-8 antigen is immunoprecipitated, the patterns of DSS-8 labeling of the embryonic nervous system indicate that there are both cell surface and extracellular forms of the antigen. The perturbation experiments have been interpreted to indicate that the extracellular form of the DSS-8 antigen is a cue guiding the growth of the median fiber tract pioneer axon along the midline. A molecule of similar function is the product of the slit gene in Drosophila which is secreted by midline glial cells and influences the path of commissural axon pathways (Rothberg et al., 1990). However, the DSS-8 antigen is not a cockroach homolog of slit because of great differences in their spatial and temporal distributions.

The DSS-8 antigen is most similar to the insect fasciclin which were also initially detected with mAbs that selectively label a subset of axons during development (Bastiani et al., 1987; Patel et al., 1987; Carr and Taghert, 1988; Harrelson and Goodman, 1988; Bieber et al., 1989; Snow et al., 1989; Grenningloh et al., 1991). Both sets of molecules appear and disappear in different parts of the developing nervous system. Both sets of molecules are not neuron specific, but are also present in specific and unusual transient patterns outside of the nervous system. However, from the details of its temporal and spatial distributions and from its molecular weight, it is clear that the DSS-8 antigen is not a homolog of any of these previously described molecules. It is ironic that this DSS-8 antigen which is so similar to the fasciclin has been shown more conclusively to play a role in guidance of pioneer axon growth than in selective axon fasciculation.

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