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Antibody to a molecular marker of cell position inhibits synapse formation in retina

(hybridoma/growth cones/embryo/cell surface/retina development)

DAVID TRISLER, JONATHAN BEKENSTEIN*, AND MATHEW P. DANIELS

Laboratory of Biochemical Genetics, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT A topographic gradient of TOP molecules in retina can be used to identify neuron position. Antibody to TOP from hybridoma cells that were injected into *in vivo* embryo eyes diffused into the retina and bound in a topographic gradient of [antibody·TOP] ([Ab·TOP]) complexes. Synapse formation in retina was inhibited in the presence of anti-TOP antibody. This suggests that TOP is involved in synapse formation and that recognition of position by neurons is necessary for normal synapse formation.

Molecular markers of cell positon have been postulated to play a role in the development of the nervous system (1). One such marker, TOP, is a cell surface molecule that is distributed topographically in a 35-fold gradient from the dorsoposterior margin to the ventroanterior margin of the chicken retina (2, 3). TOP molecules are present on most or all cells in the retina. The number of TOP molecules detected per cell varies continuously along the axis of the antigen gradient. Thus, TOP can be used to identify cell position along that axis of the retina. In the present study, the role of molecular markers of cell position in the development of the nervous system was examined by using a monoclonal antibody to TOP. Antibodies provide a means of blocking molecular function (4–7), and antibodies to neuronal surface molecules have been used to disturb growth cone behavior and disrupt neurite outgrowth (8–14).

Our objectives were to determine the accessibility of TOP in the *in vivo* retina to antibody, to determine the persistence of antibody in the retina after injection into the embryo, and to identify changes in the development of retina that is continuously exposed to anti-TOP antibody. Antibody injected extraembryonically into the amniotic cavity of young embryos *in ovo* and injected intraocularly into older embryos diffused into the retina and formed [antibody·TOP] ([Ab·TOP]) complexes in the expected gradient. Synapse formation in retina was inhibited in the presence of [Ab·TOP] complexes.

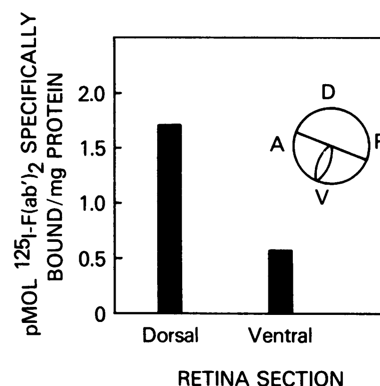

FIG. 1. Anti-TOP antibody distribution in retinas of 3-day chicken embryos (*Gallus gallus*) 1 day after injection of the antibody into the amniotic cavity. A window 25 mm in diameter was cut in the shell at the blunt end of each egg 2 days after fertilization. The inner shell membrane was removed. Mouse ascites fluid (50 μl) containing anti-TOP or P3X63 Ag8 myeloma antibody (6 mg of antibody per ml), 5×10^5 hybridoma cells that produce anti-TOP antibody, or P3X63 Ag8 myeloma cells in 50 μl of medium A [described previously (2) except without aminopterin] containing the respective antibodies were injected into the amniotic cavity of the embryos using a 25-gauge needle. The ascites fluid and the fetal bovine serum in medium A were incubated at 56°C for 30 min to inactivate complement. The shell window was sealed with cellophane tape and the eggs were incubated at 38°C in humidified air. Twenty-four hours later retinas were removed and cut into dorsal and ventral sections as shown. Symbols A, D, P, and V correspond to anterior, dorsal, posterior, and ventral, respectively. The choroid fissure, through which axons exit and enter the retina, shown extending from the ventroanterior margin to the center of the retina, was used as a landmark for dissection. Retina cells were mechanically dissociated in phosphate-buffered saline (PBS) and washed three times with 150 μl of solution B (1 mg of gelatin per ml of PBS) at 4°C. The presence of anti-TOP antibody on the retina cells was detected as described (2). Retina cells (50–150 μg of protein) were suspended in 50 μl of solution B containing 440 nM ^{125}I-labeled F(ab')$_2$ [^{125}I-F(ab')$_2$] fragment of rabbit antibody directed against mouse IgG heavy and light chains (5×10^4 cpm) and 500 μg of bovine serum albumin and incubated at 4°C for 30 min. Pellets were washed four times as described above, and radioactivity was determined. Protein was determined by a modification of the method of Lowry *et al.* (15). Specifically bound ^{125}I-F(ab')$_2$ per mg protein [i.e., pmol of ^{125}I-F(ab')$_2$ bound in the presence of anti-TOP antibody minus pmol of ^{125}I-F(ab')$_2$ bound in the presence of P3X63 Ag8 antibody] is shown on the ordinate and the retina section is shown on the abscissa. Each point is the mean determination from two embryos.

RESULTS

[Ab·TOP] Distribution After Extraembryonic Injection. Antibody to TOP injected into the amniotic cavity of *in ovo* chicken embryos 2–4 days after fertilization was detected on retina cells 1 day after injection. The concentration of [Ab·TOP] complexes detected was higher in dorsal retina than in ventral retina (Fig. 1). Antibody injected into the amniotic cavity of embryos older than 4 days and antibody injected into the yolk, a rich source of maternal antibody for the chicken (16), of 3-day embryos was not detected in retina (not shown). Thus, TOP antigen in retinas of 4-day embryos and younger is accessible to antibody from an extraembryonic source.

[Ab·TOP] Gradient After Intraocular Injection. Anti-TOP antibody was injected intraocularly into the vitreal space of

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Abbreviation: [Ab·TOP], [antibody·TOP].

*Present address: University of Virginia, School of Medicine, Charlottesville, VA 22908.

day 7–19 embryos. Retinas from the injected eyes were cut into eight sections as shown in Fig. 2 and cells from each section were assayed for [Ab·TOP] complexes. A dorsoposterior → ventroanterior gradient of [Ab·TOP] complexes of the same magnitude and orientation detected previously by *in vitro* binding studies (2) was present in retina 24 hr after intraocular injection of mouse ascites fluid containing anti-TOP antibody into day-11 chicken embryos (Fig. 2). Little or no antibody was detected bound to retina cells from the opposite noninjected eyes and from eyes injected with P3X63 Ag8 antibody. A gradient of anti-TOP antibody was present in retina 24 hr after intraocular injection for all embryonic ages tested up to day 18. Anti-TOP antibody was not detected in retina 24 hr after intraocular injection of day-18 and older embryos (not shown).

Distribution of [Ab·TOP] in Retina Cell Strata. Distribution of [Ab·TOP] complexes in the cell soma and cell process strata of retina after intraocular injection was determined in day-17 embryo retina 6 days after injection. Indirect immunofluorescence revealed that most anti-TOP antibody in chicken embryo retina was distributed in the outer and inner synaptic layers and the ganglion cell axon layer of retina after injection (Fig. 3*A*). Little antibody was detected in retinas from eyes injected with P3X63 Ag8 antibody (Fig. 3*B*). The nuclear stain was seen in the absence of antibody with rhodamine-conjugated avidin alone (not shown). These results show that anti-TOP antibody diffuses from the vitreal space, penetrates the entire thickness of retina, and binds most abundantly in the cell process layers of retina.

Duration of [Ab·TOP] Gradient. A gradient of [Ab·TOP] complexes was detected in retina 24 hr after intraocular injection of mouse ascites fluid containing anti-TOP IgG1 and persisted for 3–4 days (Fig. 4*A*). The [Ab·TOP] gradient was maintained for 9–10 days when hybridoma cells that synthesize anti-TOP antibody were injected (Fig. 4*B*). The hybridoma cells provide a continuous source of antibody for long-term maintenance of [Ab·TOP] complexes in the retina.

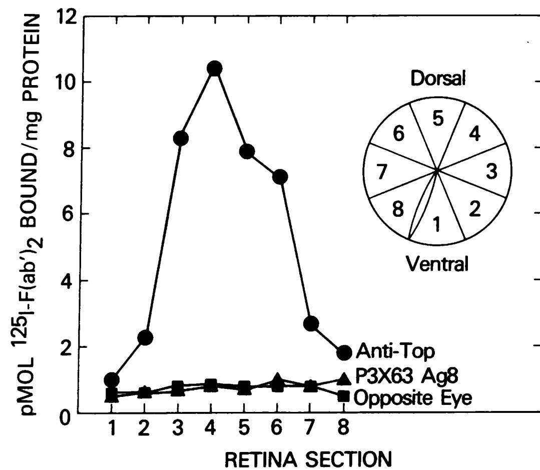

FIG. 2. [Ab·TOP] gradient in retina of 12-day chicken embryos 1 day after intraocular injection of antibody. Eggs containing 11-day embryos were cooled to 21°C, windows were cut as above, 10 μl of ascites fluid containing anti-TOP or P3X63 Ag8 antibody was injected into the vitreal space of the eye, and the embryos were incubated as above. Twenty-four hours later retinas were removed and cut into eight sections as shown and the presence of antibody on the retina cells was determined as above. ●, Retinas from eyes injected with anti-TOP antibody; ▲, retinas from eyes injected with P3X63 Ag8 antibody; ■, retinas from noninjected eyes opposite the anti-TOP injected eyes. Each point is the mean of six determinations. A detailed protocol for intraocular injection of antibody and hybridoma cells is found in ref. 10.

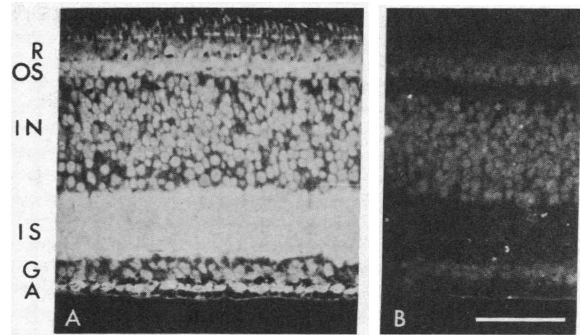

FIG. 3. Indirect immunofluorescence analysis of the distribution of antibody in 17-day chicken embryo dorsoposterior retina 6 days after intraocular injection of anti-TOP antibody-producing hybridoma cells (*A*) and of P3X63 Ag8 antibody-producing myeloma cells (*B*). R, photoreceptor cell layer; OS, outer synaptic layer; IN, inner nuclear layer; IS, inner synaptic layer; G, ganglion cell layer; A, ganglion cell axon layer. (Bar = 50 μm.) Ten microliters of medium A containing antibody and 5×10^4 hybridoma or myeloma cells was injected into the vitreal space of eyes of day-11 embryos. Six days later retinas were removed and prepared for immunohistochemical localization of *in situ* anti-TOP and P3X63 Ag8 antibody and for electron microscopy by fixation in 2.5% glutaraldehyde in 0.12 M sodium cacodylate buffer (pH 7.3) for 10 min at 21°C and then 1 hr at 4°C. Retinas were postfixed in 1.0% osmium tetroxide in 0.12 M sodium cacodylate buffer (pH 7.3) for 1 hr at 4°C, dehydrated in ethanol, and embedded in Epon 812. Distribution of antibody in the retina was detected in 1-μm-thick Epon sections. Epon and osmium were removed from the sections with sodium ethoxide/ethanol and hydrogen peroxide as described (17, 18). Epon sections (1 μm-thick) mounted on glass slides were heated at 60°C for 48 hr. Epon was removed from the tissue by incubation in saturated sodium ethoxide/absolute ethanol, 1:2 (vol:vol), for 15 min at 21°C. The slides were rinsed three times in absolute ethanol and immersed in 0.2% hydrogen peroxide for 5 min at 21°C. The tissue sections were washed three times for two min each in solution B and then one time in solution B containing 2% fetal bovine serum for 1 hr at 21°C. Sections were incubated in solution B containing biotin-conjugated anti-mouse IgG prepared in goats (E-Y Laboratories, San Mateo, CA) diluted 1:10 and 10 μg of bovine serum albumin per ml and 2% fetal bovine serum in PBS for 1 hr at 21°C. Sections were washed in solution B four times for five min each. They then were incubated in rhodamine-conjugated avidin (E-Y Laboratories) diluted 1:20 in solution B for 30 min at 21°C. The sections were washed as above, mounted with phosphate-buffered glycerol (BBL), examined on a Zeiss Universal microscope with epi-fluorescence illumination, and photographed (Kodak ASA 400 Ektachrome color slide film).

Stability of the TOP Gradient in the Presence of Antibody. The stability of TOP expression in retina after intraocular injection of anti-TOP hybridoma cells and P3X63 Ag8 myeloma cells was determined by measuring the amount of antibody from the injected source that was bound to the retina cells 5–11 days after injection and the total amount of TOP detectable in the same retina by *in vitro* assay with additional exogenous antibody. A gradient of TOP molecules was detected at all ages tested after injection (Fig. 5). Eleven days after injection, when anti-TOP antibody no longer is present in retina, a normal gradient of TOP was detected by *in vitro* assay with exogenous antibody (Fig. 5*A*). The difference in amount of anti-TOP antibody bound to retina cells in the presence of injected antibody alone and injected antibody plus exogenously added antibody may reflect the loss of injected antibody from the cells during the course of incubation of duplicate samples of cells with exogenous antibody or may represent a compartment of TOP molecules in retina that is not accessible to injected antibody. The reduction in binding of anti-TOP antibody per mg of retina protein between embryonic day 15 and day 21 (days 5 and 11 after injection) occurs normally in development (2). A normal gradient of TOP was present in retina after intraocular

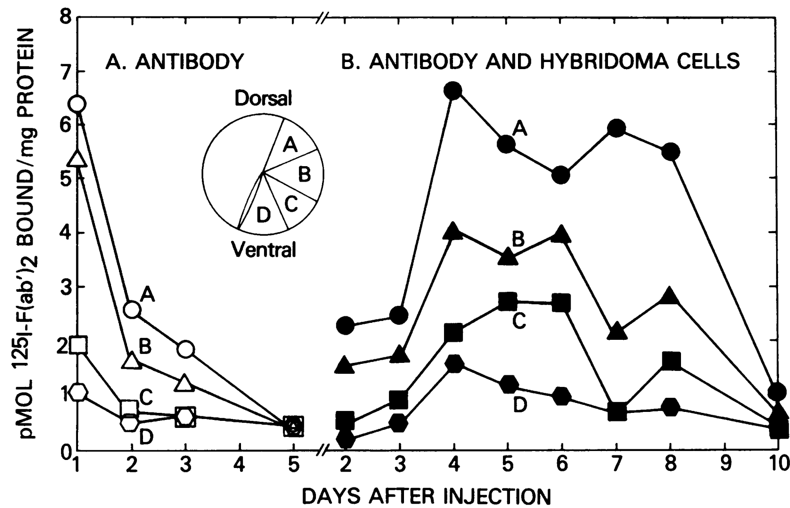

FIG. 4. Duration of [Ab·TOP] gradient in retina after intraocular injection of mouse ascites fluid containing anti-TOP antibody (*A*; open symbols) and hybridoma cells producing anti-TOP antibody (*B*; closed symbols) into 11-day chicken embryos. The presence of anti-TOP antibody in the retina was determined as described in the legend to Fig. 1. One day after injection of the antibody, and each day subsequently through day 10, retinas were removed from the embryos; one-half of each retina was prepared for electron microscopy (described in the legends to Figs. 3 and 6) and synapse counts (Fig. 7) and the other half retina was cut into four sections as shown and assayed for anti-TOP antibody. Bound ^{125}I-F(ab')$_2$ (pmol/mg of protein) is shown on the ordinate and the number of days of incubation of the embryos after injection is shown on the abscissa. ○ and ●, section A, dorsoposterior retina; △ and ▲, section B, posterior retina; □ and ■, ventroposterior retina; ⬡ and ⬢, ventral retina. Each point is the mean of two to four determinations.

injection of P3X63 Ag8 cells at all ages tested from day-15 to day-21 embryos, from day 5 to day 11 postinjection (Fig. 5*B*), and in day-7 posthatch chicken retina 19 days after intraocular injection of anti-TOP antibody (not shown). Thus, the presence of anti-TOP and P3X63 Ag8 antibody in retina does not prevent expression of the TOP antigen gradient.

Time Course of Synapse Formation in the Presence of Antibody. Antibody was injected into eyes of day-11 embryos at the time neuron process layers are forming and 2 days before the first structurally identifiable synapses appear (19–21). Two components of the process of synapse formation—the disappearance of growth cones and the appearance of synapses—were studied in retina from embryos exposed to anti-TOP antibody, P3X63 Ag8 antibody, 57D8 antibody, and

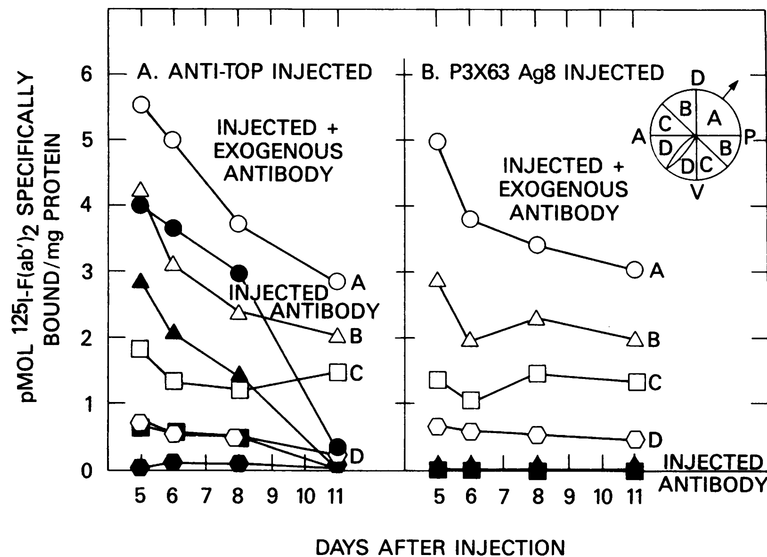

FIG. 5. Expression of TOP in retina after intraocular injection of hybridoma cells producing anti-TOP antibody (*A*) and myeloma cells producing P3X63 Ag8 antibody (*B*) into 10-day chicken embryos. Five days after intraocular injection, and on each subsequent day through 11 days, retinas were removed from the embryos, cut into sections as shown, and assayed for TOP and [Ab·TOP] complexes. Cells from corresponding sections of retina were pooled into A, B, C, and D. Cells in half of each pool were assayed for TOP by incubation with exogenously added anti-TOP antibody and half were assayed for antibody bound to the retina cells from the injected source in the vitreous. The assay conditions for TOP antigen were as described (2). Half of each pool of cells was incubated 1 hr at 4°C in buffer B containing anti-TOP antibody and the other half was incubated in buffer B without antibody. The total antibody bound to the retina cells was then detected with ^{125}I-F(ab')$_2$ as in Fig. 1. ○ and ●, retina section A; △ and ▲, retina section B; □ and ■, retina section C; ⬡ and ⬢, retina section D. The closed symbols represent specific binding of ^{125}I-F(ab')$_2$ to retina cells in the presence of anti-TOP antibody from the intraocular injected source [i.e., pmol of ^{125}I-F(ab')$_2$ bound in the presence of anti-TOP antibody minus pmol of ^{125}I-F(ab')$_2$ bound in the presence of P3X63 Ag8 antibody]. One-half picomole of ^{125}I-F(ab')$_2$ bound per mg of retina protein in the presence of Ag8 antibody. The open symbols represent the total specific binding of ^{125}I-F(ab')$_2$ in the presence of injected and exogenously added anti-TOP antibody in *A* and specific binding of ^{125}I-F(ab')$_2$ in the presence of injected P3X63 Ag8 antibody and exogenously added anti-TOP antibody in *B*.

no antibody. For this study growth cones were defined as bodies containing large, irregular membrane cisternae or vesicles (22, 23). These bodies were larger in section than most neurites and sometimes were seen in continuity with neurites and filopodia. Anti-TOP antibody from the injected hybridoma source reached maximum binding in the retina 4 days after injection (day-15 embryo). At this time, retinal development appeared normal. Electron microscopic analysis revealed that the number of growth cones and synapses and the amount of extracellular space between neurites was similar in retinas exposed to anti-TOP antibody and to no antibody (Figs. 6 *A* and *B* and 7). However, with continued exposure to anti-TOP antibody retinal development was altered.

In normal retinal development from embryonic day 15 to day 18 the number of growth cones decreased 50% (Figs. 6*C* and 7*A*), whereas the number of growth cones increased 50% during the same developmental period in retinas exposed to anti-TOP antibody (Figs. 6*D* and 7*A*) and then from 7 to 10 days after injection decreased to three growth cones per 100 μm^2, near control levels (not shown after day 19). The number of synapses increased 150% in normal retinas from embryonic day 15 to day 18 and then plateaued at 39 or 40 synapses per 100 μm^2 between day 18 and 21 as described (21); however, in the presence of anti-TOP antibody, the number of synapses increased minimally between day 15 and 18 (Figs. 6*D* and 7*B*). Then from day 18 to day 21 the number increased to 35 per 100 μm^2, approaching control levels.

Extracellular space between neurites also was affected by anti-TOP antibody. The amount of extracellular space between neurites remained unchanged from 4 to 10 days after injection of anti-TOP antibody when neurite packing normally increases. Maintenance of a large extracellular space in retinas exposed to anti-TOP antibody was restricted to the neuron process layers. No enlargement of extracellular space was detected in the cell soma layers of retina at these ages (not shown). The width of the retina cell body layers and neurite layers was normal after intraocular injection of hybridoma cells. No cell death was detected in retina from 4 to 10 days after injection of anti-TOP hybridoma cells. There was no evidence that inhibition of synapse formation was restricted to any cell type. Both conventional synapses and bipolar cell ribbon synapses were present from day 5 to 7 after injection of anti-TOP antibody. Synapse formation appeared to be interrupted across the entire inner synaptic layer in dorsal and ventral retina. No differences in development were detected between retinas from noninjected eyes and from eyes injected with myeloma cells that produce P3X63 Ag8 antibody, which binds uniformly in all regions of retina in low abundance, and 57D8 antibody, which binds in high abundance to the surface of cell soma and neurites in all regions of the retina (not shown). Retinal development, thus, appeared normal in the presence of control antibodies and the changes detected in the presence of anti-TOP antibody were inhibition of neurite development and synapse formation.

DISCUSSION

Anti-TOP antibody was introduced into the retinas of developing chicken embryos by injection of antibody or hybridoma cells into the amniotic cavity at early developmental ages and into the vitreal space of the eye at later ages up to 18 days *in ovo*. In all cases, the antibody bound to the retina cells in a topographic gradient, but the system of intraocular injection of hybridoma cells offered the advantage of providing a continuous source of antibody for maintenance of [Ab·TOP] complexes throughout the developmental period of interest.

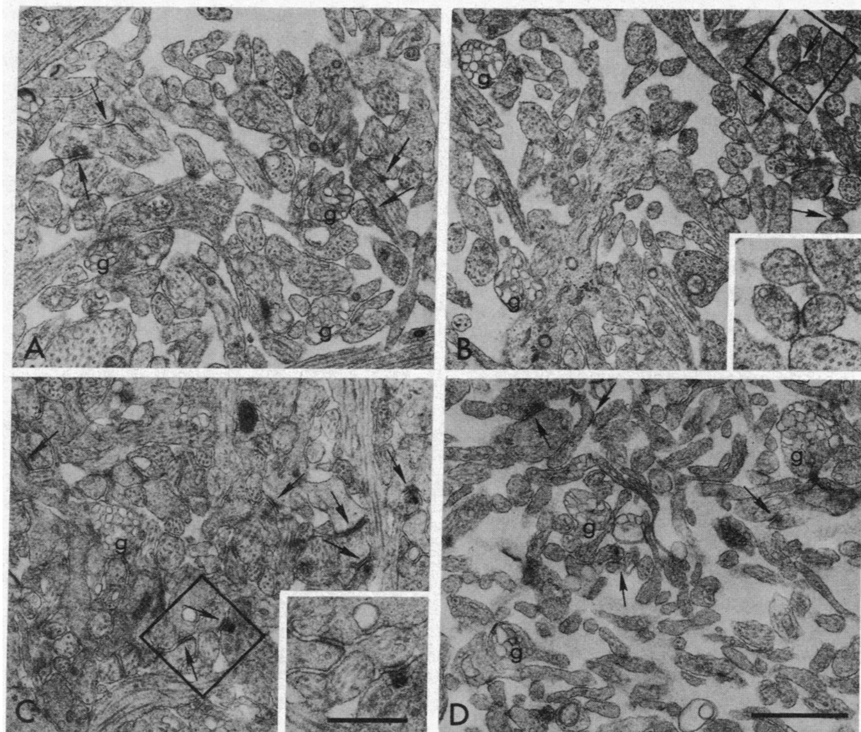

FIG. 6. Inner synaptic layer of 15-day (*A* and *B*) and 18-day (*C* and *D*) chicken embryo retina 4 and 7 days, respectively, after intraocular injection of hybridoma cells producing anti-TOP antibody (*B* and *D*) and myeloma cells producing P3X63 Ag8 antibody (*A* and *C*). Half-retinas (from embryos used in Fig. 4) were prepared for electron microscopy as described (Fig. 3). Ultrathin sections (light gold interference color) from comparable regions near the dorsoposterior and ventroanterior margins of retina from eyes injected with anti-TOP and P3X63 Ag8 were mounted on mesh grids with a carbon film and stained with uranyl acetate and lead citrate. Growth cones (g) and synapses (arrows) can be seen. (*Inset* in *B*) Enlargement of an area containing a synapse in day-15 retina. (*Inset* in *C*) Enlargement of an area containing two synapses, one a ribbon synapse of a bipolar cell. (Bar in *D* = 1.0 μm; bar in *Inset* = 0.5 μm.)

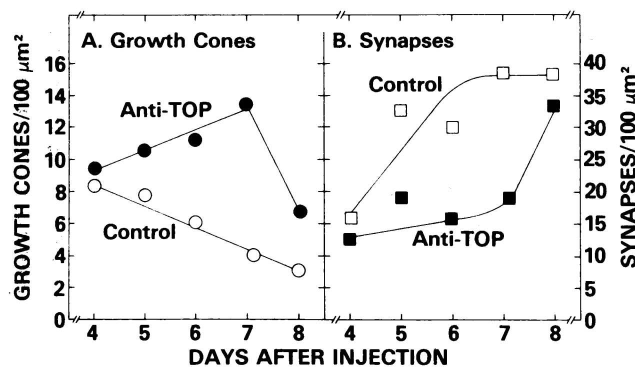

FIG. 7. Time course of synapse formation in the inner synaptic layer of retina after intraocular injection of hybridoma cells producing anti-TOP antibody, 57D8 antibody, and myeloma cells producing P3X63 Ag8 antibody into 11-day embryos. Growth cones and synapses were counted on electron micrographs of retinas (e.g., Fig. 6) from embryos in which the duration of [Ab·TOP] complexes was measured in Fig. 4. ●, Growth cones; ■, synapses in the presence of anti-TOP antibody; ○, growth cones; □, synapses in the presence of control and no antibody. The number of growth cones per 100 μm^2 and the number of synapses per 100 μm^2 are shown on the ordinates of *A* and *B*, respectively. Each point represents 55–300 growth cones (total counted, 1469) and 204–868 synapses (total counted, 4523) from one to eight retinas. Controls include regions of retina comparable to anti-TOP injected retinas from eyes injected with P3X63 Ag8 myeloma cells, the noninjected opposite eyes, noninjected eyes opposite those injected with anti-TOP hybridomas, and eyes injected with 57D8. The numbers of growth cones in experimental and control retinas were significantly different at days 5, 6, and 7 (*P* values, Student's *t* test: day 5, $P < 0.05$; day 6, $P < 0.01$; day 7, $P < 0.01$) and the number of synapses were significantly different at days 6 and 7 (day 6, $P < 0.05$; day 7, $P < 0.01$).

This strategy should prove useful in studying the roles of other cell surface molecules in retinal development.

Several groups have demonstrated effects of antibodies against nervous system molecules on the development of nerve cells and tissues *in vivo* and *in vitro*. Antibodies against chicken cognin and neural cell adhesion molecule (N-CAM) have been shown to inhibit cell–cell adhesion (24, 25) and to disrupt axonal fasciculation and the projection of most retinal ganglion cell axons on the optic tectum (12, 13), although some axons in direct contact with tectal cells correct the disruption (12). Antibody T61/3/12 blocks neurite outgrowth of chicken retina cells *in vitro* (8), whereas antibody to Thy-1 stimulates neurite outgrowth of rat retina ganglion cells *in vitro* (11). Antibody L1 inhibits granular cell migration in rat cerebellum explants (26).

We could not demonstrate inhibition of cell–cell adhesion with anti-TOP antibody or inhibition of neurite outgrowth *in vitro* in cocultures of retina cells and hybridoma cells (not shown). Retina cells were not killed during *in vitro* coculture with hybridoma cells. No evidence of cell death was seen by electron microscopy in retinas exposed to anti-TOP antibody for 11 days after injection, the longest time tested. A normal gradient of TOP molecules was detected in retina as [Ab·TOP] complexes for 9–10 days after injection. After [Ab·TOP] complexes no longer were detected, a normal gradient of TOP molecules was present. The decrease in [Ab·TOP] complexes in retina after day 18 represents the loss in accessibility of TOP to the intraocular hybridoma antibody source and not down-regulation of TOP antigen. Thus, retinas appeared normal after intraocular injection of anti-TOP antibody except for extracellular space between neurites, an increased number of growth cones, and inhibition of synapse formation, suggesting that TOP is involved in synapse formation.

Our working hypothesis is that TOP molecules mark cell position in the retina and that in the presence of [Ab·TOP] complexes, growing neurites fail to detect the TOP gradient. This could result in the prolonged presence of growth cones, since neurites fail to find their targets, and delayed synapse formation. Near-normal numbers of synapses, however, are formed eventually after exposure to anti-TOP antibody. Further work is required to determine whether the synaptic connections that occur in retina after intraocular injection of anti-TOP antibody are positionally correct.

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