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Isolation of the Progenitor Cells of the Sympathoadrenal Lineage from Embryonic Sympathetic Ganglia with the SA Monoclonal Antibodies

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Our previous articles in this series described the production of five monoclonal antibodies (SA1–5) that bind to adrenal chromaffin cells and to cells in embryonic sympathetic ganglia and adrenal primordia (Carnahan and Patterson, 1991), and the downregulation of the sympathoadrenal (SA) antigens *in vivo* as neuronal markers begin to be expressed (Anderson et al., 1991). These results support the hypothesis that sympathetic neurons and adrenal chromaffin cells are derived from a common embryonic progenitor that displays both neuron- and chromaffin cell–specific markers. We have taken advantage of the fact that at least some of the SA antigens are expressed on the cell surface to isolate SA$^+$ cells from embryonic day 14.5 rat superior cervical, sympathetic ganglia by fluorescence-activated cell sorting. This population of cells is significantly enriched in the expression of markers (tyrosine hydroxylase and neurofilament) found in the putative progenitors *in situ*. Growth in glucocorticoid maintains the expression of the SA antigens in the sorted cells and induces the chromaffin cell marker enzyme phenylethanolamine *N*-methyl transferase. In contrast, growth of the sorted cells in basic fibroblast growth factor, NGF, and insulin results in the rapid loss of SA1 expression and the outgrowth of neurites. The ability to manipulate the fate of the SA$^+$ cells *in vitro* confirms the suggestion from the *in vivo* observations that the SA$^+$ cells in the ganglia are at least bipotential progenitors, capable of differentiating along the chromaffin or neuronal pathways.

The three derivatives of the sympathoadrenal (SA) lineage, sympathetic neurons, small intensely fluorescent (SIF) cells, and chromaffin cells, are all derived from a common source, the neural crest (Weston, 1963; Le Douarin and Teillet, 1971, 1974; Pearse et al., 1973). While these derivatives can be distinguished by a number of morphological and molecular criteria, they can be interconverted using the appropriate developmental signals. Doupe et al. (1985b) showed that SIF cells isolated from neonatal rat sympathetic ganglia can give rise to neurons in the presence of NGF or to chromaffin cells in the presence of a high concentration of glucocorticoid. Fully differentiated chromaffin cells from neonatal or adult rats can also be converted into neurons in the presence of NGF (Unsicker et al., 1978; Doupe et al., 1985a); in this case, chromaffin cells are converted into neurons by first going through an SIF cell–like intermediate stage. These results suggested that all three derivatives might be derived from a common progenitor *in vivo* (Landis and Patterson, 1981; Doupe et al., 1985a).

This hypothesis was further supported by the findings of Anderson and Axel (1986) in rat, and Vogel and Weston (1990) in chick, who demonstrated that neuronal markers are expressed by cells migrating from embryonic sympathetic ganglia into the adrenal gland and that these markers are lost as chromaffin cells differentiate. This suggested that the same progenitors may give rise to both neurons and chromaffin cells. In fact, Anderson and Axel (1986) were able to isolate from embryonic adrenal medullae progenitor cells that can differentiate into sympathetic neurons or chromaffin cells, depending on the presence of NGF or glucocorticoid, respectively. Seidl and Unsicker (1989) obtained a similar result using a different cell isolation approach.

Not only are neuronal markers expressed by cells in the embryonic adrenal medulla, but conversely, chromaffin cell markers are found, early in embryonic development, on virtually all cells of sympathetic ganglia (Anderson et al., 1991). These results raise the possibility that both adrenal medulla and sympathetic ganglia initially contain bipotential progenitor cells that transiently coexpress both neuronal and chromaffin cell markers. Moreover, these markers are eventually expressed in a mutually exclusive manner as the cells further differentiate along one or the other pathway. We here use three of the SA monoclonal antibodies described in an accompanying article (Carnahan and Patterson, 1991) to isolate by fluorescence-activated cell sorting (FACS) a bipotential progenitor population from embryonic sympathetic ganglia. These cells acquire chromaffin cell properties when cultured in medium containing glucocorticoid and acquire neuronal properties when cultured in medium containing basic fibroblast growth factor (bFGF), NGF, and insulin. These data further support the idea that chromaffin cells and sympathetic neurons derive from a common embryonic progenitor and indicate that this precursor initially coexpresses markers characteristic of two of its differentiated derivatives.

Materials and Methods

Preparation of cells for sorting. Dissection of 12 timed pregnant rats was necessary to obtain enough cells to be plated after FACS. At early stages of development [embryonic day 14 (E14)], the sympathetic gan-

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Table 1. Intracellular versus cell surface SA staining in developing sympathetic ganglia

	% of cells positive for SA					
	E11.5	E13.5	E14.5	E16.5	E18.5	P0
Intracellular SA1	100	55–60	25–30	15–20	3–5	<1
Surface SA(1,2,4)	0*	3–5	8–15	15–20	30	5–10

Cells from ganglia of various ages were dissociated and either stained with the antibody mixture SA(1,2,4) as living cells (Surface SA), or fixed and permeabilized and stained with SA1 (Intracellular SA). The exception (*) is E11.5 where surface staining was done at E12.5 and showed no staining even then. Because E11.5 ganglia could not be dissected free of other tissues, the intracellular staining data were obtained from frozen sections double stained with TH. The data are the ranges of percentages of TH^+ cells that are SA^+ (from two independent experiments). P0, day of birth.

glia are barely larger than the sympathetic chain of which they are a part. To ensure that we were indeed dissecting superior cervical ganglia, the first group of dissected ganglia was frozen and sectioned, and positive staining for both tyrosine hydroxylase (TH) and the SA1–5 antibodies was confirmed. The standard preparation involved dissecting 250–300 superior cervical ganglia from E14.5 embryos that were collected in L-15–air plating medium (Hawrot and Patterson, 1979). Ganglia were transferred with a Pasteur pipette into a sterile, siliconized 1.7 ml microcentrifuge tube (PGC Scientifics), washed in calcium/magnesium-free Hanks' balanced salt solution (GIBCO), resuspended in 1 mg/ml collagenase (Worthington Biochemical Corp.), and incubated for 30 min at 37°C. The cells were dissociated using gentle pipetting with siliconized pipette tips (PGC Scientifics). The cells were then washed in L-15–air, 10% fetal calf serum (FCS); resuspended in 100 μl of 50% FCS, 50% SA(1,2,4) (1:1:1) antibody mixture in hybridoma medium; and incubated 30 min at room temperature in the same microcentrifuge tube. One milliliter of L-15–CO_2 (Hawrot and Patterson, 1979) was added to dilute the antibodies, and the cells were centrifuged for 5 min at 100 × *g*. The pellet was washed again using the same procedure. A fraction of the dissociated cells was used as a negative control in which hybridoma medium was substituted for the antibody mixture. Both control and SA^+ cells were resuspended in a mixture of 50% FCS, 10% biotinylated horse anti-mouse (Vector) antibodies at a final dilution of 1:100, in a volume of 100 μl. After 30 min incubation at room temperature, the cells were washed once with L-15–CO_2 plus 5% FCS and twice with L-15–CO_2 without serum. They were then incubated in 100 μl of a filtered solution of streptavidin conjugated to fluorescein isothiocyanate (Amersham). After 20 min at room temperature, cells were washed and filtered through a sterile nylon mesh to remove clumps.

These steps were performed under sterile conditions, and the tubing in the cell sorter itself was rinsed with sterile PBS. At this stage of development, about 8–10% of the ganglionic cells display detectable surface staining using the mixture of three antibodies. We routinely used the 6–7% brightest-staining cells (see Fig. 3).

Plating the sorted cells. Many different culture conditions and substrata were tested for their efficacy in supporting the attachment and growth of the sorted cells. Substrata included various combinations of laminin, polylysine, fibronectin, collagen, Matrigel, extracellular matrix secreted by bovine corneal endothelial cells, and Vitrogel, as well as cells from the Schwannoma cell line P2TD3, the muscle cell line L6, and primary heart cells. The growth factors tested in various combinations included primary heart cell–conditioned medium, insulin, NGF (7S, 1 μg/ml; Hawrot and Patterson, 1979), FGF (R & D Systems; 10 ng/ml), insulin (Sigma; 2.5 μg/ml, fresh solution for each experiment), dexamethasone (Sigma; 10^{-6} M), extract of rat embryo sympathetic chain, chick embryo extract, fetal calf serum, and adult rat serum. The most favorable surfaces were air-dried collagen (with cells plated at very high density) and primary rat heart cells treated with mitomycin C (to prevent further division). Heart cell coculture yielded good attachment even at low cell plating density (300 cells per 10 mm well). The drawback of the heart cell coculture condition was that about 50% of the plated cells extended neurites after 10 hr in the absence of added growth factors. For the collagen surfaces, 100,000 SA^+ cells were plated into four wells of 2 mm diameter.

Hearts from E18 embryos were dissected and enzymatically dissociated in 1 mg/ml collagenase dissolved in Hanks' buffer, pH 7.4. After 30–40 min at 37°C, cells were dissociated by gentle trituration and washed several times in L-15–CO_2 medium containing 5% rat serum. The plating density was calculated to cover ¾ of the surface of the collagen-coated Aclar well (Hawrot and Patterson, 1979). The heart cells were grown for 2–3 d to form a monolayer, treated with mitomycin C (Sigma) at 10 μg/ml for 1 hr at 37°C, and rinsed carefully with L-15–CO_2 prior to plating the sorted SA^+ cells.

Cells were stained and counted as described in an accompanying article (Carnahan and Patterson, 1991).

Results

Intracellular staining with the SA antibodies develops before cell surface staining

Virtually all TH^+ cells in early (E11.5) sympathetic ganglia are positive for all five SA antibodies, and this staining appears to be cytoplasmic (Carnahan and Patterson, 1991). Cell surface staining with these antibodies develops more gradually (Table 1); at E15.5, SA1 and SA2 stain only about 15% of freshly dissociated, unfixed cells (the procedure for surface staining). Staining by SA4 and -5 appears about E16.5, and SA3 staining begins on E17, while SA3 never seems to stain the cell surfaces well at all. Curiously, as the surface staining becomes apparent, cytoplasmic staining begins to decline (Table 1). Thus, 30% of cells from E18.5 ganglia are positive for surface staining with SA(1,2,4), while only 3–5% of the cells display cytoplasmic staining for SA1 (Table 1). It is of interest that those cells that express detectable surface SA(1,2,4) also express the highest levels of staining with anti-TH antibodies (Fig. 1). The weak surface staining of cells from early ganglia raises difficulties for using the SA antibodies to sort cells on the FACS. However, since distinct epitopes are recognized by the various SA antibodies (Carnahan and Patterson, 1991), several of the antibodies can be used together in order to enhance labeling. Using SA(1,2,4), we demonstrated clear surface staining of about 5% of the cells in E13.5 sympathetic ganglia even though at this stage no detectable surface staining is apparent using each of the antibodies separately. Double staining of this sorted population with anti-TH and anti-neurofilament (NF) antibodies reveals that virtually all of the cells express each of these antigens as well as very strong intracellular staining for all of the SA antibodies (data not shown). Nonetheless, the absolute number of cells obtained from sorting E13.5 ganglia is quite small, so E14.5 ganglia were used for further study.

Characterization of cells from E14.5 sympathetic ganglia

Dissociated cells from E14.5 sympathetic ganglia were characterized by double labeling with several different markers. The SA1 antibody can be used as a chromaffin cell marker in this lineage (Carnahan and Patterson, 1991), anti-NF antibodies [140 kDa (Dräger et al., 1983)] are used to better visualize neurite-bearing cells, the B2 antibody is used to identify a neuronal precursor (Anderson and Axel, 1986; Anderson et al., 1991), and anti-TH antibodies distinguish early sympathetic neurons and chromaffin cells from glial cells and fibroblasts. Dissociated

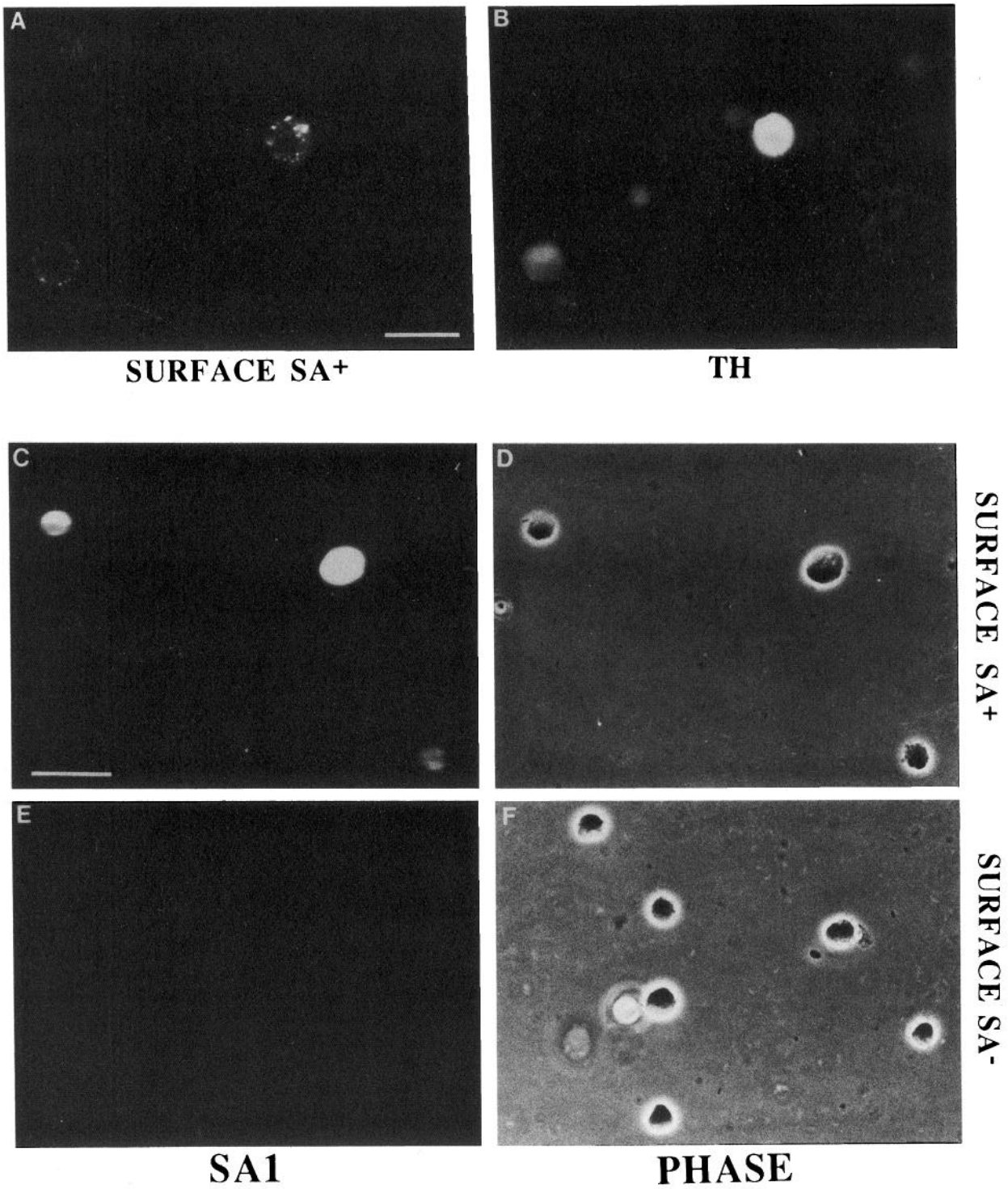

Figure 1. Surface SA$^+$ cells display bright TH staining. Cells from E14.5 day superior cervical ganglia were dissociated, surface stained with the SA(1,2,4) antibody mixture (*A*), and then fixed, permeabilized, and double stained for TH (*B*). Nearly all SA$^+$ cells are brightly stained for TH. These cells were also separated into SA(1,2,4)$^+$ and SA(1,2,4)$^-$ fractions by FACS, as described for Figure 2, and then fixed, permeabilized, and stained for intracellular SA1 (*C, E*). As demonstrated quantitatively in Table 2, most of the cells in the SA(1,2,4)$^+$ fraction are SA1$^+$ (*C, D*), while most of the cells in the SA(1,2,4)$^-$ fraction are SA$^-$ (*E, F*). Scale bars: *A*, 50 μm for *A* and *B*; *C*, 30 μm for *C–F*.

cells were stained with these markers before and after separation on the FACS using the SA(1,2,4) antibody mixture. As summarized in Table 2, the SA1$^+$ population is composed of cells that are highly enriched for NF, TH, and SA1 intracellular staining. This population represents 30–40% of the cells in the ganglia at this stage in development. About 10% of the SA$^+$ cells also express the B2 marker, a finding consistent with double staining *in situ* (Anderson et al., 1991). The TH staining of the B2$^+$ cells is much less strong than the rest of the SA$^+$ population, which is consistent with the idea that B2 labels a more advanced stage

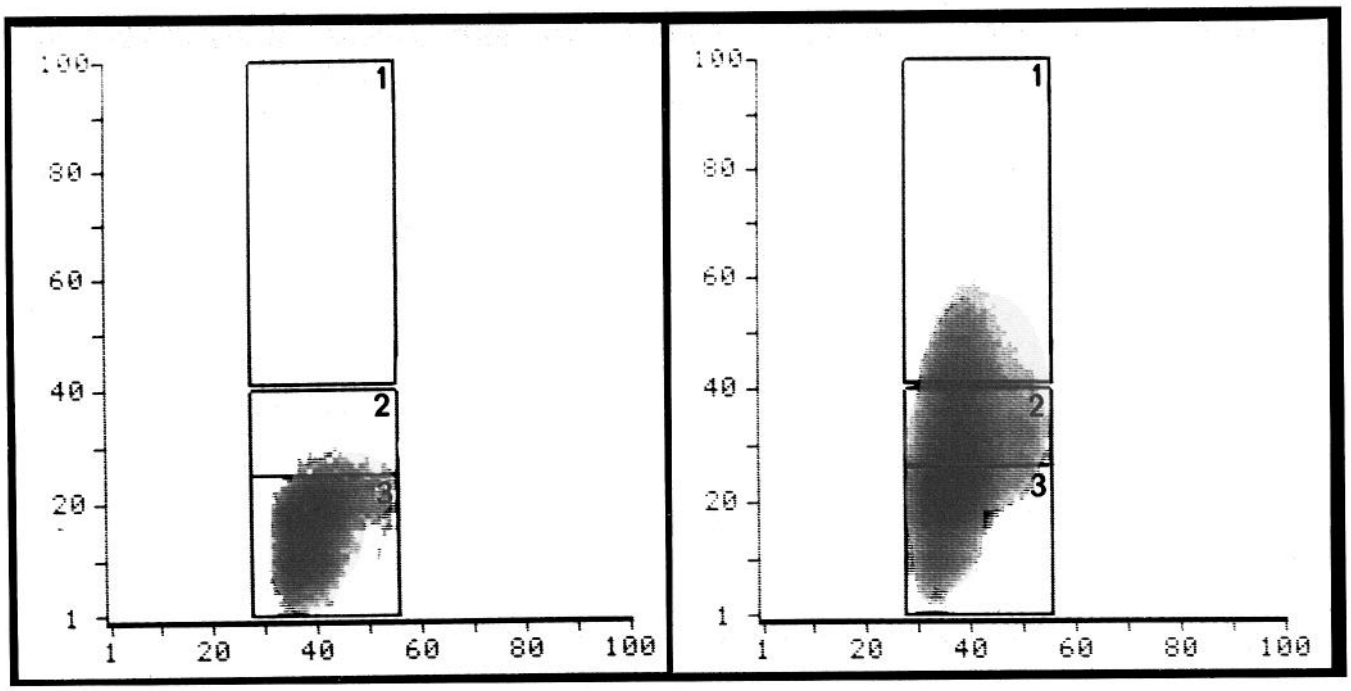

Figure 2. FACS profiles of E14.5 ganglion cells using the SA(1,2,4) antibody mixture. E14.5 superior cervical ganglia were dissociated, incubated with either the SA(1,2,4) antibody mixture or culture medium, and subjected to FACS. Plotted on the *ordinate* is relative fluorescence intensity, and on the *abscissa* is the relative size of the cells. The *left panel* is a control for nonspecific binding of the fluorescence-labeled secondary antibody, in which cells were incubated with culture medium. The *right panel* shows the profile of cells incubated with the SA antibodies. Cells in *box 1* were used for further experiments as the SA^+ fraction; these represent the 5–7% brightest-staining cells. Cells corresponding to those in *box 3* were used in some experiments as the SA^- fraction.

of differentiation in the neuronal pathway (Anderson and Axel, 1986). Therefore, it was possible to divide the cells of E14.5 ganglia into four groups: (1) $SA1^+$ TH^+ NF^+ $B2^-$, (2) $SA1^+$ TH^+(weak) NF^+ $B2^+$, (3) SA^- TH^+ NF^+ $B2^+$, and (4) $SA1^-$ TH^- NF^- $B2^-$.

Growth and differentiation of $SA1^+$ cells in culture

Sorting SA^+ cells. For each experiment, the cells from about 300 E14.5 sympathetic ganglia were dissociated and stained with the SA(1,2,4) antibody mixture. A typical FACS profile is shown in Figure 2; the upper boxes indicate the population used as the SA^+ fraction. These cells expressed the highest level of SA surface staining and represented about 7% of the original 10^6 cells.

Growth of sorted cells on collagen. The SA^+ cells attached poorly to the usual air-dried collagen surface, so extreme caution was necessary in moving the dishes for microscopic observation. In addition, the cells had to be plated at high density, and the growth factors under study, bFGF, NGF, and insulin or dexamethasone, had to be added from the start so that the medium was not changed at later times. At 10 hr after plating, nearly all cells were small and round; few flat cells could be identified (Table 3). Many more flat cells were observed in the SA^- population, and some of the cells in this population extended neurites spontaneously (data not shown). SA^+ cells treated with bFGF, NGF, and insulin displayed no obvious morphological differences from those treated with dexamethasone at this stage.

Table 2. Antigenic characterization of E14.5 sympathetic ganglion cells

	% of cells positive for markers			
Cells	TH	B2	SA1	NF
Unsorted	50	ND	27	50
Sorted				
$SA(1,2,4)^+$	95	10	90	92
$SA(1,2,4)^-$	35	ND	8	35

Cells of E14.5 sympathetic ganglia were dissociated and either stained immediately with various antibodies or separated by FACS after incubation with the SA(1,2,4) mixture, and subsequently stained for the other markers. Staining with the B2 antibody was carried out on living cells without fixation. Staining with the TH, SA1, and NF antibodies was carried out on aliquots of the cells after fixation and permeabilization; these data are for cytoplasmic staining. The numbers are expressed as a percentage of the unsorted, SA^+, or SA^- populations. Thus, for example, 27% of the total population is positive for cytoplasmic SA1 staining, while 90% of the cells expressing SA(1,2,4) on their surfaces are positive for cytoplasmic SA1 staining. Since separation of the SA^+ and SA^- populations is not perfect, the numbers from the top row are not identical to those that can be computed from the bottom two rows. The values shown are from a single experiment and are representative of five such experiments. ND, not done.

Table 3. Development of SA$^+$ cells in the absence of other cells

	Dexamethasone	bFGF, NGF, insulin
10 hr postplating		
Spherical cells	297	396
Flat cells	6	34
48 hr postplating		
Chromaffin-like cells	262	14
Neuron-like cells	2	822
Flat cells	64	99

SA$^+$ cells were obtained by dissociating E14.5 sympathetic ganglia, separating the cells by FACS, and plating them on collagen-coated dishes in the presence of bFGF, NGF, and insulin, or dexamethasone. At 10 hr postplating, the cells were analyzed by phase microscopy, and all of the cells were placed in one of the two categories. After 48 hr, the cells were characterized again and grouped according to their morphology and, in some experiments, their immunohistochemical markers. Chromaffin cells were characterized as being small round cells that maintain SA staining and express high levels of TH staining. This type of cell also expresses PNMT staining in cultures that were carried for 5 d in dexamethasone. Cells characterized as neurons lost SA staining, grew NF$^+$ neurites, and displayed low levels of TH staining. These data are from one set of dishes that was analyzed at 10 and again at 48 hr postplating. Therefore, the protein growth factors stimulated mitosis as the total cell numbers increased from 430 to 835. In dexamethasone, in contrast, the total cell number remained approximately constant.

Cells were observed 48 hr after plating by phase-contrast microscopy, fixed, and stained for NF and SA1. Although there were only two dishes for each growth condition, the experiment was reproduced five times, yielding a similar difference each time between the dishes treated with dexamethasone versus bFGF, NGF, and insulin. The results shown in Table 3 are important because the number and morphology of the cells at the beginning of the analysis (10 hr postplating) were very similar in the two conditions. In dexamethasone, cell number did not change significantly by 48 hr, nor did the morphology of most of the cells. They also continued to express SA1 and TH at the high levels appropriate for chromaffin cells (Fig. 3). In some cases, cell attachment was maintained for 5–7 d. When these cultures were stained with an anti-phenylethanolamine *N*-methyl transferase (PNMT) antibody, many of the cells grown in dexamethasone displayed high levels of this chromaffin cell antigen (Fig. 4*B*), demonstrating their complete differentiation into chromaffin cells. There was an increase in the number of flat cells, but this was somewhat variable between experiments. Process outgrowth by the SA$^+$ cells is minimal in the presence of dexamethasone (Fig. 5*B*). In contrast, glucocorticoid did not block process outgrowth by the SA$^-$ cell population (cultured separately; Fig. 5*C*). This is consistent with the notion that loss of SA expression connotes further progression along the neuronal differentiation pathway.

In contrast, a dramatic change occurred in the cells grown in the protein growth factors. Virtually all of the originally SA$^+$ cells began growing neurites, lost their SA1 staining, and expressed lower levels of TH staining (Fig. 3*B,D,F*). Unlike the cells grown in steroid, NF expression was maintained (Fig. 5). The protein growth factors also stimulated mitosis, approximately doubling cell number during this period. The mitotic effect was also apparent in the formation of colonies of cells. The increase in cell number was not enough to explain the phenotypic conversion, however. That is, a small minority of cells did not multiply at very high frequency and grow neurites. On the contrary, most of the initially round, SA$^+$ cells were observed to extend neurites in the protein growth factors. Thus, we conclude that most of the SA$^+$ population either can maintain the rounded morphology and SA1 staining characteristic of chromaffin cells and acquire the PNMT chromaffin cell marker, or can respond to protein growth factors by losing SA1 staining, growing neurites, and maintaining NF expression characteristic of neurons.

One interesting and consistent finding in the dishes treated with the protein growth factors was the presence of small groups of round cells without neurites, still weakly stained for SA1. It is possible that these cells represent the SIF lineage developing in a predominantly neuronal environment.

Culture on heart cells. The SA$^+$ cells from E14.5 ganglia attach to heart cell monolayers in rat serum and in the absence of added growth factors. Therefore, this paradigm has an advantage over that in the previous set of experiments where cells were initially plated into medium containing protein growth factors or dexamethasone. After 10 hr postplating, the cocultures were examined by immunohistochemistry, and the cells were characterized as belonging to one of three categories: flat, neuron-like, or chromaffin-like. The data (Table 4) are presented as the percentage of the cells in each dish belonging to each category so that the data from three separate experiments could be combined. Flat cells were primarily SA$^-$ TH$^-$ NF$^-$. This population was composed of heart cells and a few contaminating

Table 4. Development of SA$^+$ cells on heart cells

	Neuron-like	Chromaffin-like
10 hr postplating		
(no growth factors)	47% ± 4	53% ± 4
3 d postplating		
FGF, NGF, insulin	65% ± 3	35% ± 3
Dexamethasone	12% ± 3	89% ± 6

SA$^+$ cells were obtained by dissociating E14.5 sympathetic ganglia, separating them by FACS, and plating them on monolayers of mitotically arrested heart cells in the absence of added protein growth factors and glucocorticoid. At 10 hr cells were analyzed for their phase microscopic appearance. Small spherical cells corresponded to cells that, in other experiments where immunohistochemistry was carried out, maintain their SA staining and express high levels of TH. These are termed chromaffin-like. Cells termed neuron-like have grown processes and correspond to cells with high levels of NF, low TH, and no SA staining. At 10 hr, these same cultures were given medium containing either dexamethasone or a mixture of three protein growth factors. At 3 d postplating, these cultures were again analyzed by phase microscopy and, in some cases, by immunohistochemistry. The results are expressed as the percentage of the spherical cells lying on top of the sparse heart cell monolayer that express each of the phenotypes. The numbers are the values obtained in three different experiments, each with duplicate dishes, ±SEM.

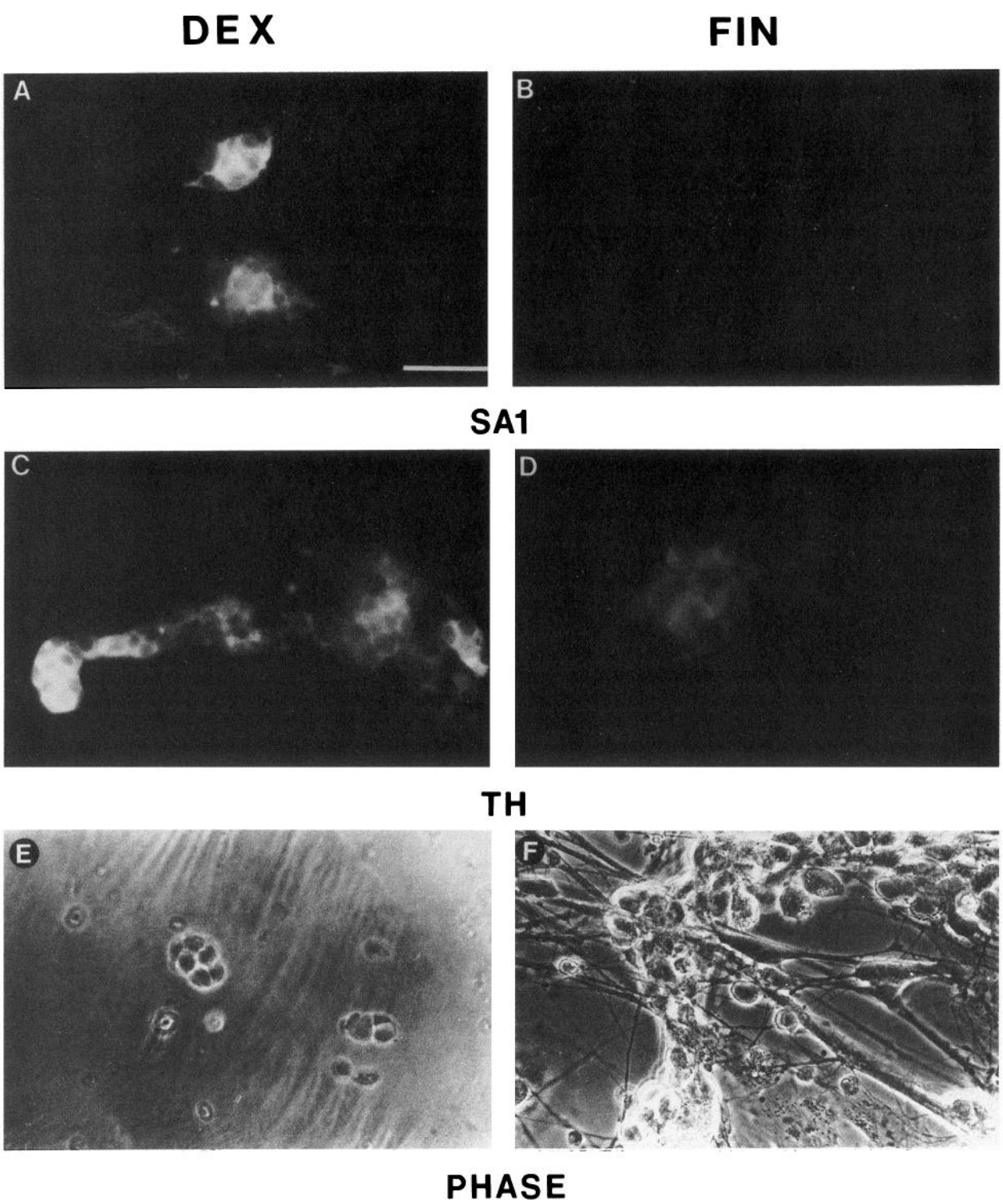

Figure 3. Environmental control of antigenic and morphologic phenotypes. After isolation by FACS, SA^+ cells were grown in either a mixture of bFGF/insulin/NGF (*FIN*; *B, D, F*) or dexamethasone (*A, C, E*). After 5 d, the cultures were fixed, permeabilized, and stained for chromaffin cell properties (intracellular $SA1^+$ and bright TH; *A, C*) or neuronal markers ($SA1^-$ and weak TH; *B; D*) as well as the presence of neurites (*F*). Scale bar, 50 μm.

SA^- cells from the sorting procedure. Spherical cells without neurites made up about half of the SA^+ cells in the absence of added growth factors (Table 4). Cells with neurites made up the other half of the non-flat cell population. When these cultures were shifted into a medium containing bFGF, NGF, and insulin and analyzed 3 d later, neurite-bearing cells made up two-thirds of the SA^+ population, and spherical cells the other third. This is the same type of shift in phenotype observed in the cultures grown on collagen, in the absence of other cell types. The major difference between the two culture conditions in the development of the SA^+ cells is that the sorted cells grow neurites in the absence of added protein growth factors when plated on

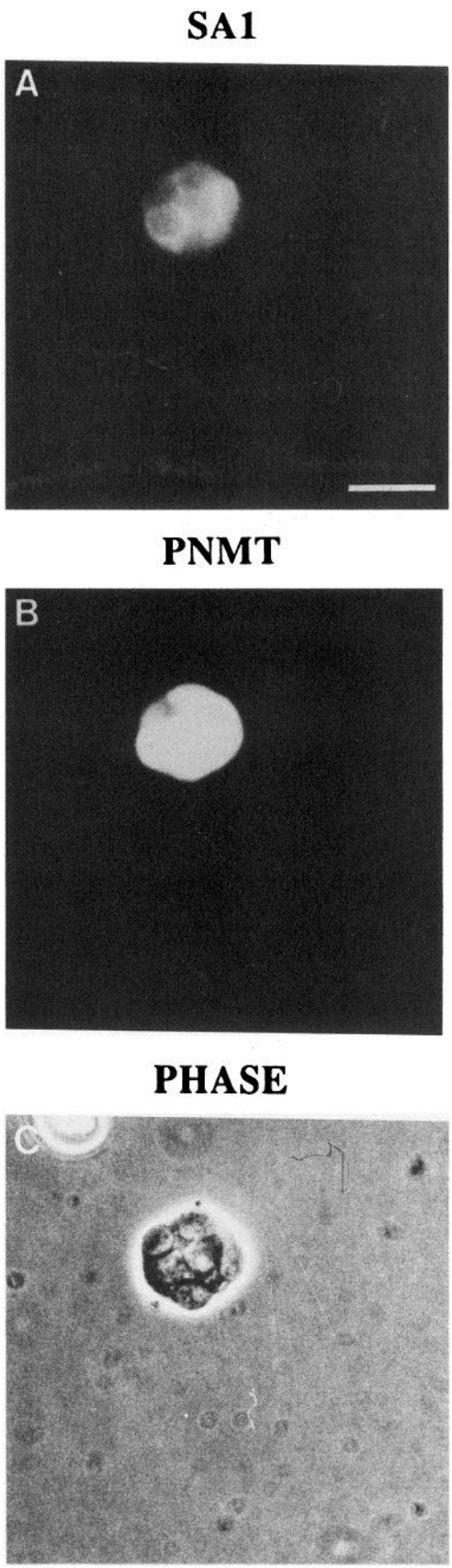

Figure 4. Glucocorticoid promotes chromaffin cell differentiation. After isolation by FACS, SA$^+$ cells were grown for 5 d on collagen-coated dishes as in Figure 3 and double stained for intracellular SA1 (*A*) and PNMT (*B*) after fixation and permeabilization. In this example, a tightly adhering group of cells are stained; nuclei are visible in *A*, but the bright staining in *B* obscures them. The majority of such cells were brightly stained by the PNMT antibody. Scale bar, 35 μm.

heart cells. In addition, dexamethasone did not strongly induce PNMT expression in the heart cell cocultures, even after 1 week in culture.

Discussion

Previous observations, both in culture and *in vivo,* provided evidence that embryonic sympathetic ganglia, like embryonic adrenal medullae, contain bipotential progenitor cells that can differentiate into neurons or chromaffin cells, depending on the environment in which they develop. We produced several monoclonal antibodies (SA1–5) that bind to antigens both in the cytoplasm and on the surfaces of the TH$^+$ cells of these ganglia (Carnahan and Patterson, 1991). The SA antibodies enabled us to isolate progenitor cells from the ganglia in order to analyze their developmental potential and to identify factors that control their phenotypic decisions in culture. Separation of the SA$^+$ cells by FACS proved important because 40% of the unfractionated cell population does not express high levels of TH and may not be part of the SA lineage. All of the sorted, SA$^+$ cells are TH$^+$ and NF$^+$. The SA$^+$ cells from E14.5 ganglia are not a homogeneous population, however. Approximately 10% of the cells are B2$^+$ and express lower levels of TH than the other SA$^+$ cells. These properties suggest that this subpopulation is further along the neuronal differentiation pathway than the rest of the cells, consistent with studies of B2$^+$ cells isolated from embryonic adrenal glands (Anderson and Axel, 1986; Anderson et al., 1991).

The cells displaying the highest levels of surface staining with a mixture of three SA antibodies were selected for growth in cell culture, and two conditions were employed. Plating the cells on collagen surfaces permitted us to study SA progenitors in the absence of other cell types, but attachment was poor. Therefore, the growth factors under study, bFGF, NGF, and insulin, or the synthetic glucocorticoid dexamethasone was added to the initial plating medium so that the cells would not be disturbed by changing the medium at a later point. In cases where the same number of SA$^+$ cells survived the initial 10 hr postplating period, it was observed that nearly all of the cells responded to each of the conditions by assuming either a neuronal (in protein growth factors) or a chromaffin (in dexamethasone) phenotype. This result is consistent with the identification of SA$^+$ cells as bipotential progenitors that can respond to either type of influence. It is also possible, however, that the use of different plating media could select predetermined subpopulations of SA$^+$ cells for differential survival. By this hypothesis, predetermined neuronal precursors would attach and survive in the protein growth factor–containing medium, and the predetermined chromaffin precursors would survive in the dexamethasone-containing medium.

To circumvent this problem, SA$^+$ cells were also plated on a more adhesive surface consisting of mitotic-arrested heart cell monolayers. In this case, added growth factors were not required during the initial culture period, so sister cultures were plated and the cells attached under identical conditions. This paradigm yielded results qualitatively similar to those obtained using the collagen surface; dexamethasone promoted the chromaffin phe-

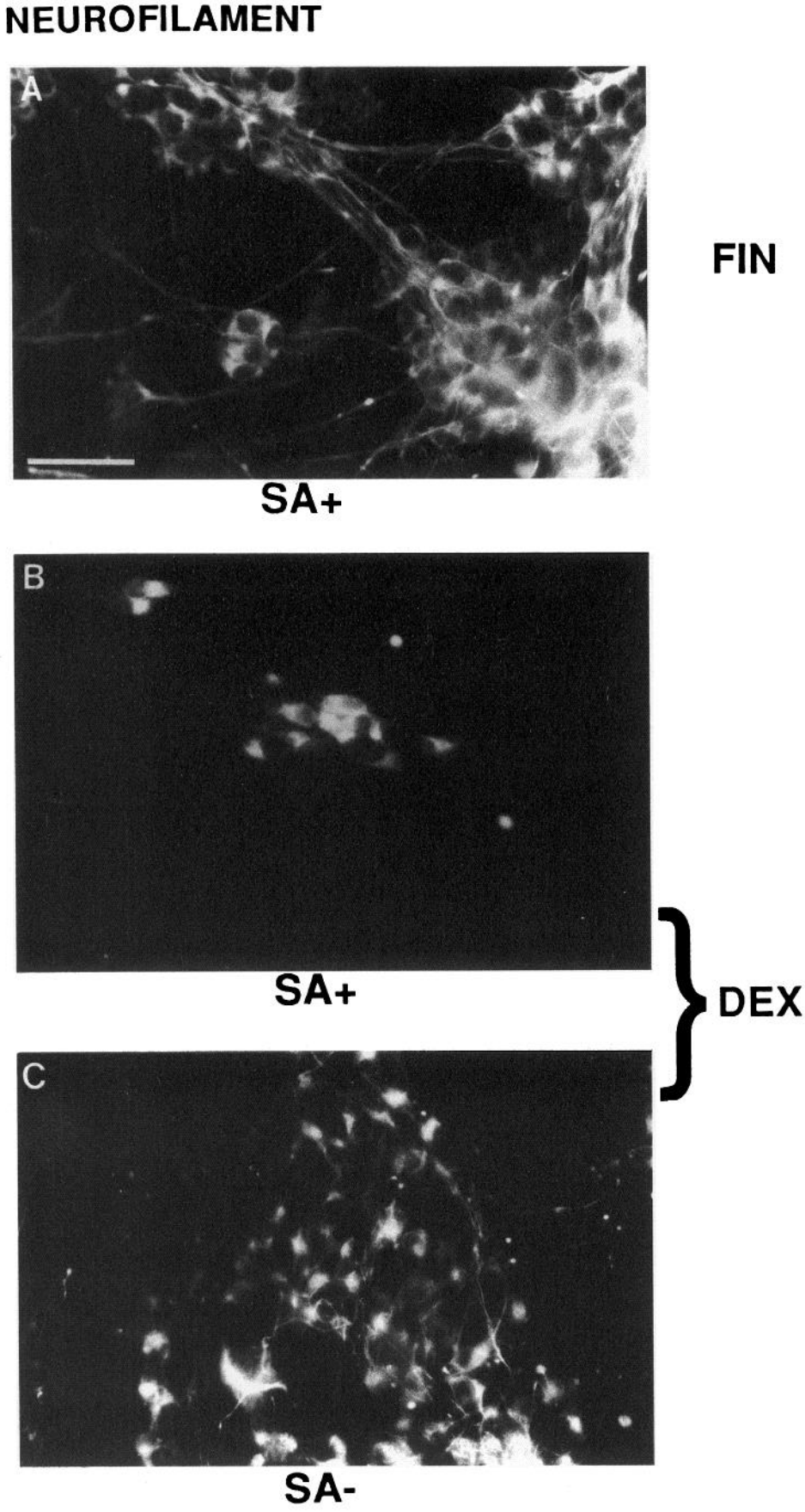

Figure 5. NF expression by SA$^+$ and SA$^-$ cells in response to protein growth factors or dexamethasone. SA$^+$ (*A, B*) and SA$^-$ (*C*) cells were isolated by FACS, grown on collagen-coated surfaces for 48 hr and stained for NF. In the presence of bFGF, insulin, and NGF (*FIN, A*), SA$^+$ cultures exhibit abundant NF$^+$ processes. Such processes are not found in SA$^+$ cultures grown in dexamethasone (*DEX, B*). In contrast, many SA$^-$ cells spontaneously grow NF$^+$ processes even in the presence of dexamethasone (*C*). These differences are quantified in Table 3. Scale bar, 50 μm.

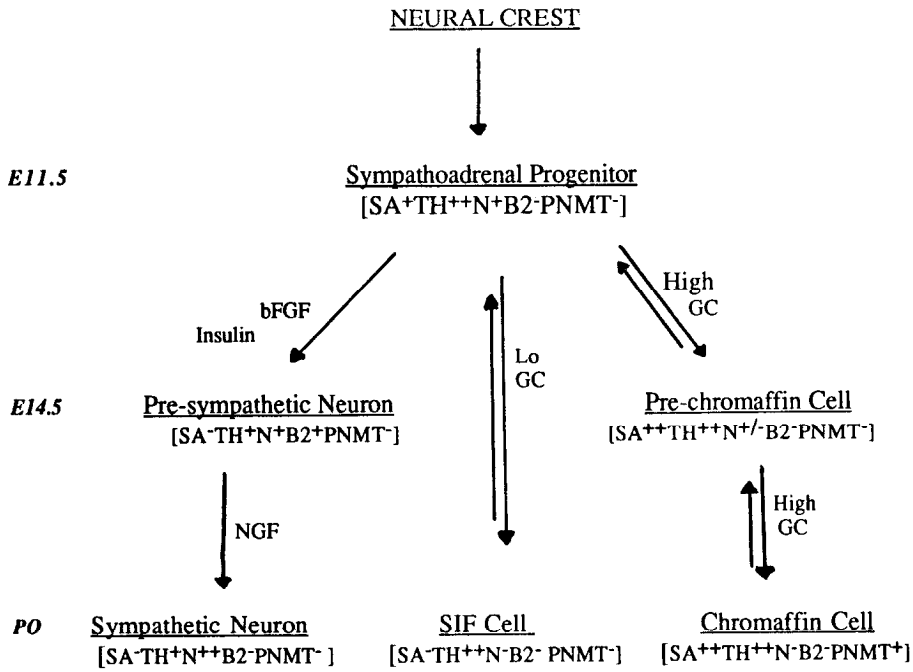

Figure 6. Diagram of the SA lineage consistent with available data. All three products of this lineage are derived ultimately from the neural crest; thus, the SA progenitor is depicted as arising from the crest. The crest also gives rise to numerous other lineages, not shown here. In addition, the number of distinct steps leading to the differentiation of the progenitor is not known but is depicted by a *single arrow.* The various cell types delineated here display characteristic sets of antigen, as described in the *brackets,* and the factors that promote differentiation along the several pathways are indicated next to the *arrows.* The stages of development corresponding to the various cell types are listed on the *left. N,* a combination of neurites, NF, and SCG10 staining; *GC,* glucocorticoid; *P0,* day of birth. All other abbreviations and symbols are as described in the text.

notype and protein growth factors promoted the neuronal phenotype. The heart cells themselves provided a neuronal differentiation influence on the plated cells, so that the quantitative results were not as decisive as those obtained on the collagen surfaces. Nonetheless, the two sets of experiments taken together provide strong support for the existence of a bipotential, SA progenitor population in embryonic sympathetic ganglia. Thus, it seems likely that a common progenitor populates both the embryonic ganglia and the adrenal gland. It is interesting that the progenitor expresses both neuronal (NF) and chromaffin (SA1–5) differentiation markers prior to assuming its final phenotype *in situ* (Anderson et al., 1991). Thus, signals in the adrenal and ganglionic environments act to downregulate markers characteristic of the phenotype that is inappropriate for each environment. This mechanism could be relevant for the plasticity seen in these derivatives in adulthood (Anderson, 1989).

The protein growth factors bFGF and NGF were chosen to promote neuronal differentiation of SA progenitors because prior work, both in culture and *in vivo,* had indicated that these proteins may play a role in neurite outgrowth in the SA lineage. Of particular relevance were the findings that chromaffin cells from neonatal rats and a SA progenitor cell line initiate neuronal differentiation in response to bFGF and subsequently develop a dependence on NGF for further maturation and survival (Stemple et al., 1988; Birren and Anderson, 1990). Insulin was also included in this mixture because it can stimulate proliferation of neuronal precursors from embryonic rat sympathetic ganglia and is necessary for the survival of postmitotic sympathetic neurons *in vitro* (Wolinsky et al., 1985; DiCicco-Bloom and Black, 1988; DiCicco-Bloom et al., 1990). The synthetic glucocorticoid dexamethasone was employed to promote the chromaffin cell phenotype because previous work established such a role for this hormone (Landis and Patterson, 1981; Anderson, 1989; Patterson, 1990). The results from the present study further support the roles suggested for these protein and steroid factors.

The evidence that the SA antibodies label both chromaffin cells and bipotential SA progenitor cells supports the notion that these two cell types are quite similar. Both cells are thought to express high levels of TH (Jacobowitz, 1970; Taxi, 1979; Taxi et al., 1983) and catecholamine histofluorescence (Owman et al., 1971; Bohn et al., 1981); both can differentiate into neurons under appropriate conditions (Unsicker et al., 1978; Aloe and Levi-Montalcini, 1979, 1980; Doupe et al., 1985a; Stemple et al., 1988); and both are thought to contain large dense-cored vesicles (Elfvin, 1967). These two cells can, however, be distinguished ultrastructurally because the progenitors are thought to contain very few dense-cored vesicles (Eränkö, 1972, 1976; Tennyson and Mytilineou, 1976; Ross et al., 1990). All of these observations are consistent with a previous suggestion that the progenitor cells are immature chromaffin cells (Anderson, 1989). The lack of PNMT in the precursor cells is not a key difference since many mature chromaffin cells lack this enzyme as well.

It was proposed previously that the progenitor cells would resemble SIF cells (Landis and Patterson, 1981) because cultured SIF cells could serve as precursors for both chromaffin cells and sympathetic neurons (Doupe et al., 1985b). The SA antibodies, however, discriminate between the precursor cells and mature SIF cells, by not binding to the latter (Carnahan and Patterson, 1991). It is interesting, in this respect, that SIF cells, identified in ganglia by high TH, are the last cells to lose SA binding (Carnahan and Patterson, 1991). This might suggest that the progenitor cells are indeed closely related to SIF cells; both have high levels of TH and catecholamines, both can differentiate into neurons, and both have large dense-cored vesicles. Prior to the availability of the SA antibodies, the only criterion for distinguishing chromaffin cells and SIF cells was the size of the vesicles; those in chromaffin cells are larger than those in SIF cells (Coupland, 1965; Taxi, 1979; Taxi et al., 1983). These considerations suggest a novel interpretation of the Doupe et al. (1985b) results: perhaps the "SIF" cell that served as a precursor to both chromaffin cells and neurons in those exper-

iments was actually the SA progenitor itself, and the markers available at that time could not distinguish mature SIF cells and the progenitors. It would be of interest to return to those experiments with the SA antibodies as tools to help identify the various cell types.

Another view of the SIF cell–progenitor relationship can be gained by considering insights from several other systems in which progenitors have been isolated from both developing and adult sources. For instance, Wolswijk and Noble (1989) found that there are differences between the O-2A progenitor cells derived from neonatal and adult rat optic nerves. The differences include antigenic phenotype, morphology, and cell cycle time. Differences have also been observed between hematopoietic stem cells isolated from embryonic yolk sac and adult bone marrow (Metcalf and Moore, 1971). Moreover, although both satellite cells from adult skeletal muscle and embryonic myoblasts can give rise to multinucleated skeletal muscle fibers, these progenitors display several differences in their phenotypes (Cossu et al., 1987). In the same way, then, SIF cells in neonatal and possibly adult ganglia could be viewed as the mature form of the SA progenitor, somewhat different from its embryonic counterpart but still able to generate the progeny of the lineage.

The SA progenitor examined here may have a broader role in autonomic development than its name implies. The observations that cells in the embryonic gut transiently express TH (de Champlain et al., 1970) and respond to NGF (Kessler et al., 1979) and glucocorticoid *in vivo* (Teitelman et al., 1979; Jonakait et al., 1980, 1981) raised the possibility that enteric neurons could pass through a noradrenergic stage before assuming the cholinergic and other phenotypes characteristic of neurons in enteric ganglia (Cochard et al., 1978; Teitelman et al., 1978; Jonakait et al., 1980). This hypothesis received considerable support from the observations of Baetge et al. (1990), who found that all cells in the fetal rat bowel that express neuronal markers also transiently express TH and dopamine β-hydroxylase. The latter catecholaminergic enzyme continues to be expressed by a subpopulation of these neurons into adulthood. Such findings suggested that enteric neurons could, in fact, be derived from the same progenitor cell as the SA derivatives (Landis and Patterson, 1981). Our data that the SA1 and B2 antibodies also stain embryonic enteric neurons expressing TH and NF (Carnahan et al., 1991) support this idea. Moreover, a common region of the neural crest (somites 5–7 in the chick; Le Douarin, 1982) gives rise to both sympathetic and enteric ganglia. It would thus be of interest to determine if the SA progenitors isolated from embryonic sympathetic ganglia or adrenal glands could be influenced to differentiate properties characteristic of enteric neurons. At present, however, the only feature that distinguishes sympathetic neurons from enteric neurons is the lack of NGF dependence in the latter population. Many transmitters and neuropeptides found in the gut can also be expressed by sympathetic neurons (Patterson, 1990).

Our present understanding of the SA lineage is summarized in Figure 6. The markers used to distinguish the various stages cell types are SA1–5, TH, B2, PNMT, and N [N refers to results with both NF and the neuron-specific protein SCG10 (Stein et al., 1988; Henion and Landis, 1990; Mori et al., 1990). The prechromaffin cell stage is identified in the adrenal gland by the maintenance of SA1 expression and lack of B2 induction. These cells are distinguishable from E11.5 SA progenitors by their reduced expression of neuronal markers ($N^{+/-}$). Presympathetic neurons are the $B2^{+}$ $SA1^{-}$ cells in sympathetic ganglia that express NF and SCG10 and have lost competence to respond to glucocorticoid (Anderson et al., 1991). The chromaffin and SIF cell phenotypes are shown as reversible because Doupe et al. (1985a,b) found that these cells could be converted into normal sympathetic neurons. Those authors also found that the chromaffin/SIF phenotypic choice could be controlled by the glucocorticoid concentration, as depicted in Figure 6. The arrow from neural crest to the SA progenitor is not meant to imply that this is a single step or that all crest cells go through this pathway.

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