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Specific neural and adrenal medullary antigens detected by antisera to clonal PC12 pheochromocytoma cells

(nerve growth factor/cell surface antigens/cell culture/immunology)

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ABSTRACT **Antisera were prepared against a clonal line (PC12) of rat pheochromocytoma cells that were grown in either the presence or the absence of nerve growth factor (NGF). PC12 cells respond to NGF by growing neurites and express many differentiated properties of adrenal chromaffin cells and sympathetic neurons. The antisera, after absorption with rat liver, kidney, spleen, and thymus, reacted in the micro-complement fixation and indirect immunofluorescence assays with PC12 cells (both NGF-treated and untreated), brain, adrenal medulla, and superior cervical ganglia, but not with a variety of other tissues. Cross-absorption of the antisera with brain and adrenal medulla indicated the presence of components that were specific to brain, adrenal medulla, and PC12 cells. Brain-specific and adrenal-medulla-specific antigens were also detected in other species, such as rabbit, cat, and mouse. No differences could be detected in the specificity of antisera raised against either NGF-treated or untreated PC12 cells. Indirect immunofluorescence on live cells also detected cell surface antigens on both cell bodies and processes. These results suggest that such antisera may be used to detect, localize, and identify specific neural and adrenal medullary antigens.**

A clonal cell line (PC12) was recently established (1) from a transplantable rat adrenal pheochromocytoma (2). These cells display differentiated properties of adrenal chromaffin cells such as synthesis (1), release, and storage (3, 4) of catecholamines. In addition, such cells acquire further neuronal properties after treatment with nerve growth factor (NGF) (5), including outgrowth of extensive neurites (1), electrical excitability, and high sensitivity to acetylcholine (6). The homogeneous nature of this system, together with its phenotypic stability and its NGF-modulatable response, offers many advantages as a model for neuronal differentiation (1). The use of immunological techniques to study neural differentiation has been shown to be quite promising. Several studies on antigens in normal brain (7–9) and on the surface components of a growing number of cell lines of neural origin (10–14) have demonstrated that the serological approach can be used to identify specific neural antigens. The present paper deals with the characterization of antisera raised against PC12 cells cultured in the presence and absence of NGF. These antisera appear to recognize antigens specific to adrenal medulla and to brain.

MATERIALS AND METHODS

Cell Culture. PC12 cells (approximately 50 to 250 generations after their original isolation) were maintained on plastic or collagen-coated tissue culture dishes or on polylysine-coated (15) cover slips as previously described (1, 3). NGF-treated cultures were obtained by maintenance for 2–3 weeks in medium supplemented with 2.5S NGF at 50 ng/ml (16). Protein levels were assayed on either whole-cell homogenates or 700 × *g* pellets as described by Lowry *et al.* (17), using a bovine serum albumin standard.

Antisera. Stationary phase PC12 cells (NGF-treated and untreated) were detached from the culture dishes with a rubber policeman in 1–4 ml of phosphate-buffered saline, pH 7.4, and washed twice by centrifugation with 12 ml of the same buffer. The cells were homogenized with a Teflon–glass homogenizer and emulsified with complete Freund's adjuvant, and approximately 1×10^7 cells were injected into the foot pads of each guinea pig. A second injection was performed a week later in the same manner except with incomplete Freund's adjuvant. Subsequent immunizations were at weekly intervals with cell homogenates and incomplete Freund's adjuvant injected subcutaneously. Blood was collected by cardiac puncture 6–8 days after the sixth and seventh immunizations. Antisera from different animals and different bleedings were not pooled.

Absorption of Antisera. Bulk absorption (18) of the antisera was performed with washed particulate fractions of homogenates of liver, spleen, kidney, and thymus of NEDH rats, the strain in which the pheochromocytoma arose (2). Antisera were heat inactivated at 56° for 30 min and then absorbed (1:2 dilution) three times for 30 min at room temperature, using equal volumes of pelleted tissue homogenates and antiserum. All antisera used for the present study were absorbed by this means. Subsequent absorptions with PC12 cells, brain, or adrenal medulla were carried out by two 1-hr incubations and one overnight incubation (with shaking at 4°) of equal volumes of antisera and pelleted homogenates.

Micro-Complement Fixation. Antiserum titers and specificities were determined by ^{51}Cr release micro-complement fixation (19, 20). For the assays, cells in monolayer cultures were washed three times with medium and then broken and homogenized by sonication. The homogenates were either assayed directly or centrifuged at 700 × *g* for 10 min and the pellet was resuspended in dextrose/Veronal buffer (Gibco).

Indirect Immunofluorescence. Indirect immunofluorescence on tissue sections was carried out with various dilutions of antisera as described previously (21). For fluorescence staining of living cells on cover slips, antisera at various dilutions were added directly to the dishes containing the cover slips. The cells were incubated for 90 min at 37° in a 93% air/7% CO_2 incubator. After the cover slips were washed three times with culture medium, unabsorbed fluorescein-conjugated goat antiserum to guinea pig immunoglobulin (Antibodies, Inc.) was added at a final dilution of 1:75. After 60 min at 37°, the cells were washed, fixed in 4% glutaraldehyde for 10 min at room temperature, and washed twice again, and the cover slips

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Abbreviation: NGF, nerve growth factor.

Table 1. Micro-complement fixation assay of AsPC12± against particulate fractions of rat tissue homogenates

Target	Titer of anti-serum*
PC12−	1:1200–1:3000
PC12+	1:1200–1:3000
Adrenal	1:640–1:1200
Brain	1:640–1:1200
Superior cervical ganglia	1:640–1:1200
Liver	<1:40
Kidney	<1:40
Thymus	<1:40
Spleen	<1:40

The experiment was repeated 4 times, each time with equal amounts of protein for each target sample. The range of protein levels for different experiments was 0.1–10 μg per sample. AsPC12± were bulk absorbed with liver, kidney, spleen, and thymus as described in the *text*.

* Final dilution of AsPC12± yielding 50% fixation of complement.

Table 2. Specificity of AsPC12± (as measured by micro-complement fixation assay) after absorption with particulate fractions of rat brain and adrenal

Tissue used for absorption of antiserum*	Titer of antiserum,† using as target‡:		
	PC12±	Adrenal	Brain
None	1:1200–1:3000	1:640–1:1200	1:640–1:1200
PC12±	<1:40	<1:40	<1:40
Adrenal	1:640–1:1200	<1:40	1:320–1:640
Brain	1:640–1:1200	1:320–1:640	<1:40
Brain and adrenal	1:320–1:640	<1:40	<1:40

* Absorption was carried out as described in the *text*, using particulate fractions.

† Titer is defined as in Table 1.

‡ Targets were particulate fractions (1.1 μg per sample) prepared as described in the *text*. Results were similar in 2 separate experiments.

containing the cells were mounted with a drop of 1:1 mixture of glycerol and phosphate-buffered saline. Observation was with a Zeiss epifluorescent microscope.

RESULTS

In the subsequent sections, the following nomenclature is used uniformly: PC12+ = NGF-treated PC12 cells; PC12− = untreated PC12 cells; PC12± = both NGF-treated and untreated PC12 cells. AsPC12+ = antiserum raised against PC12+; AsPC12− = antiserum raised against PC12− cells. The term AsPC12± is used when identical responses were seen with both AsPC12+ and AsPC12−.

Specificity of Antisera. After bulk absorption with non-neuronal rat tissue, AsPC12± had an antigenic titer between 1:1200 and 1:3000 against PC12+ and PC12− cell homogenates as measured by micro-complement fixation (Table 1). Neither the antisera (at 1:40 dilution) nor the homogenates (up to 25 μg per sample) by themselves fixed complement. Also, neither the culture medium, nor NGF (at 50 ng/ml), nor the collagen crossreacted with the antisera. The particulate and supernatant fractions of PC12 homogenates (prepared by a 100,000 × *g* centrifugation for 1 hr) were assayed by micro-complement fixation and each fraction was recognized to the same extent on a per mg protein basis by both the bulk-absorbed AsPC12+ and AsPC12− antisera.

To analyze whether the bulk-absorbed antisera recognized antigens shared by other rat tissues, particulate fractions of various organs were tested as targets (Table 1). Of the tissues tested, only brain, adrenal, and superior cervical ganglia shared antigenic specificities with PC12±. Various brain regions (cerebrum, cerebellum, midbrain, and brain stem) showed no differences in recognition by AsPC12±. Liver, kidney, spleen, and thymus showed no cross-reactivity. Controls of tissues (1–10 μg of protein) without antisera did not fix complement.

Species Specificity. AsPC12± recognized adrenal medulla and brain from mouse, rabbit, and cat, but not from chicken, in both micro-complement fixation and indirect immunofluorescence assays.

Specificity of Organ-Specific Antigens. Cross-absorption studies were carried out to see whether the adrenal and neural components recognized by AsPC12± were shared between these organs or unique to each of them. AsPC12± were absorbed with either rat adrenal or brain and then tested against homogenates of PC12±, adrenal, or brain, using the micro-complement fixation assay (Table 2). AsPC12± absorbed with adrenal no longer recognized adrenal homogenates but still recognized homogenates of brain and PC12±. Similar results were obtained with AsPC12± absorbed with brain; i.e., the antisera no longer recognized brain but still recognized homogenates of adrenal and PC12±. Finally, AsPC12± absorbed with both adrenal and brain did not recognize either adrenal or brain but still recognized PC12± cells. As a negative control, AsPC12± absorbed with PC12± did not recognize PC12± cells, adrenal, or brain. These experiments suggest that AsPC12± recognizes three classes of components: one that is unique to the adrenal and to PC12 cells, another that is unique to the brain and to PC12 cells, and a third that is limited to PC12 cells.

Localization of Antigens on Rat Brain and Adrenal. Indirect immunofluorescence localization of antigens recognized by both AsPC12± was performed on 10-μm-thick frozen tissue sections, using fluorescein-conjugated goat anti-guinea pig immunoglobulin (dilution 1:75) as the second antibody. In the adrenal, specific staining was limited to the medulla (Fig. 1*A*). Controls in the same tissue performed with bulk-absorbed pre-immune serum or with AsPC12± absorbed with PC12± cells or with adrenal showed no staining (Fig. 1*B*). The indirect immunofluorescent technique also detected antigens on sections of brain. Staining was to numerous neural components and was present throughout various brain regions. On a sagittal section of cerebellum, for example, staining was rather generalized with more staining evident on grey than on white matter (Fig. 1*C*). Control antisera absorbed with PC12± or with brain were also negative (Fig. 1*D*).

The results with indirect immunofluorescence employing cross-absorbed AsPC12± paralleled those obtained by micro-complement fixation. Both AsPC12± absorbed with adrenal medulla did not stain the adrenal medulla but still stained the brain, though staining was weaker than with unabsorbed serum at the same dilution. The weakness of the staining could be due to loss of antibodies either by nonspecific adsorption or by specific absorption with antigens common to adrenal medulla and brain. Similarly, AsPC12± absorbed with brain still yielded specific indirect immunofluorescent staining of adrenal medulla.

Expression of Surface Antigens on PC12 Cells. Indirect immunofluorescence was performed on PC12± cells in culture. Both AsPC12+ and AsPC12− (at a final titer of 1:200) stained the cell bodies of PC12± as well as the neurites of PC12+ cells (Fig. 2). AsPC12± absorbed with either PC12+ or PC12− cells gave no detectable fluorescence staining. Because significant

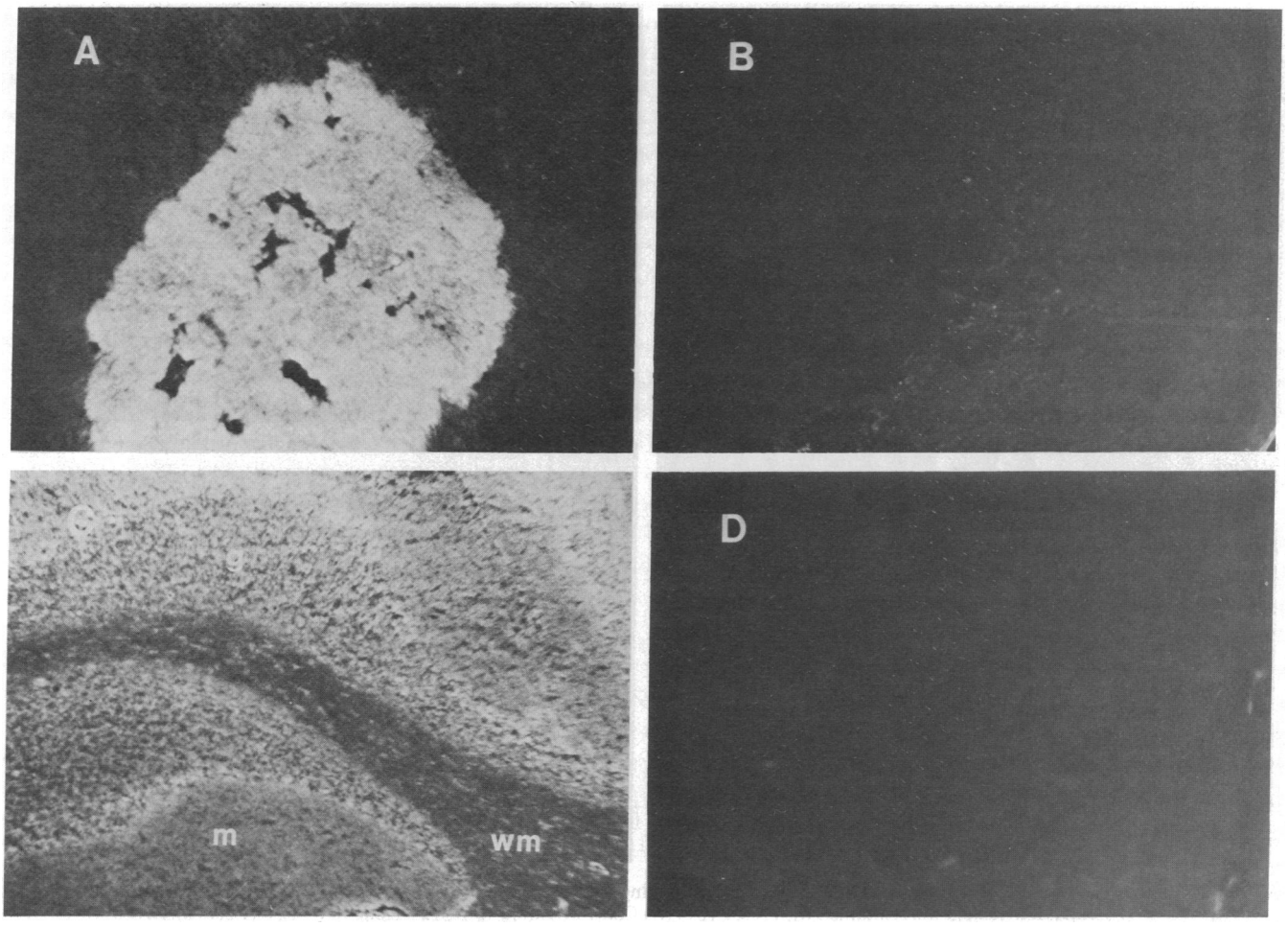

FIG. 1. Indirect immunofluorescence localization of antigens in rat adrenal and cerebellum with AsPC12+. (All × 192.) (*A*) Cross section of rat adrenal. Immunofluorescence localization with AsPC12+ (1:160) is limited to medulla. Cortex does not show staining. Comparable results were obtained with AsPC12−. (*B*) Control staining of adrenal section with pre-immune serum (1:160). (*C*) Sagittal section of rat cerebellum with AsPC12+ (1:160). The pattern of fluorescence shows weak staining of white matter (wm), generalized staining of molecular (m) and granule-cell (g) layers, and apparently more intense staining at the interface of molecular and granule-cell layers. Comparable results were obtained with AsPC12−. (*D*) Control staining of cerebellar section with pre-immune serum (1:160).

levels of antibodies would not be expected to enter living cells, such findings indicate that AsPC12± contain antibodies directed against surface antigens of PC12± cells. To further substantiate that the staining was indeed on the surface, antisera against brain filament and tubulin (kindly provided by R. Liem and S.-H. Yen) were used as controls. Neither of these antisera stained living PC12± cells. However, when PC12± cells were fixed and dehydrated, the antisera stained the interiors of cell bodies and processes. Experiments were also carried out with AsPC12± absorbed with adrenal medulla, brain, or both adrenal medulla and brain. All three absorbed antisera still stained the cell bodies of living PC12± cells and the neurites of living PC12+ cells, though twice the concentration (1:100 dilution) was required to yield the same intensity.

Reproducibility of Antisera. AsPC12± prepared in eight different guinea pigs and with PC12± ranging between approximately 50 and 200 generations after their original isolation have thus far been found to exhibit identical properties after bulk absorption.

DISCUSSION

In the present study, antisera raised in guinea pigs against PC12 rat pheochromocytoma cells and then bulk absorbed with particulate material from a variety of rat internal organs, appeared to recognize three distinct classes of antigens. Among the tissues tested, one class of antigens is present in neural tissue and a second is present in adrenal medulla. A third set of antigens is limited to PC12± cells, among the cells studied thus far. Indirect immunofluorescence experiments on living PC12± cells indicate that at least one of these components is exposed on the cell surface. Preliminary cytotoxicity and indirect immunofluorescence data further suggest that AsPC12± also recognize determinants that are exposed on the surface of cultured rat sympathetic neurons.

The variety of cell-specific antigens that AsPC12± might be expected to recognize is closely linked with the properties of the PC12 line. As pheochromocytoma cells, PC12 cells are the neoplastic counterparts of adrenal medullary chromaffin cells. PC12 cells also resemble sympathetic neurons in that they respond to NGF (but to no other factor thus far tested) by neurite outgrowth. In addition, the line expresses differentiated properties found in both chromaffin cells and neurons—such as synthesis, storage, and release of neurotransmitters, chemosensitivity, and electrical excitability. Finally, because PC12 cells are neoplastic and display an elasticity with respect to their state of differentiation, they might also be expected to express

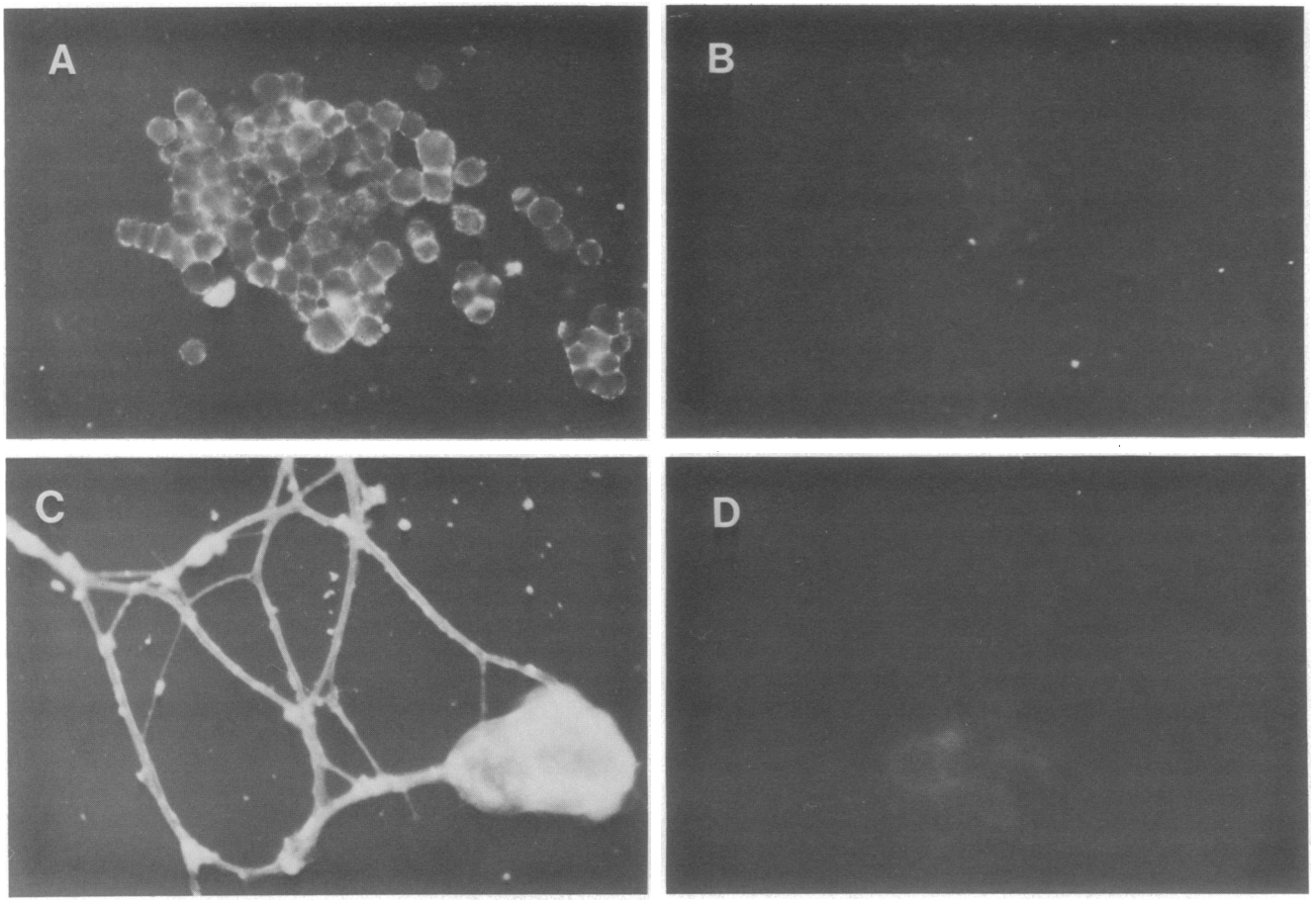

FIG. 2. Indirect immunofluorescence localization of antigens on living PC12± cells with AsPC12+. (All × 319.) (*A*) Staining of PC12− using AsPC12+ (1:200). Comparable results were obtained with AsPC12−. (*B*) Control staining of PC12− using AsPC12+ (1:200) absorbed with PC12− cells. (*C*) Staining of PC12+ using AsPC12+ (1:200). Note staining of both cell bodies and processes. Comparable results were obtained with AsPC12−. (*D*) Control staining of PC12+ using AsPC12+ (1:200) absorbed with PC12+.

antigens characteristic of developing tissues to which they are related, such as neural crest.

Although PC12+ and PC12− show marked differences in their morphologies, we did not detect differences either in the properties of antisera raised against the two types of cultures or, by the use of such antisera, in the antigenic composition of the cells themselves. Such results are in keeping with recent findings that the protein composition of PC12 cells undergoes few major changes in response to NGF (22) and are consistent with the possibility that fiber outgrowth entails mainly a redistribution and/or reorganization of molecules that are already present in the cell. Recent cytotoxicity experiments in our lboratory have provided some preliminary evidence for such a redistribution of antigens on the cell bodies and processes of NGF-treated cells. The lack of antigenic distinction found here between PC12− and PC12+ cells is furthermore consistent with the observations that PC12− cells are highly differentiated with respect to many properties even before exposure to NGF. It is also of interest to note, in contrast, that previous studies have suggested that morphological differentiation of C1300 murine neuroblastoma cells (triggered by a variety of treatments, but not by NGF) is accompanied by changes in protein composition (23) and in antigenic properties (11).

Our results indicate that antisera raised against PC12 cells may be useful in a variety of ways. For example, it should be possible to use the antisera to detect, localize, and, ultimately, isolate antigens that are specific to a single or to a small number of cell types (e.g., neurons or chromaffin cells). It should also be of interest to follow the appearance (and possibly disappearance) of such markers during development. The utility of the antisera for such objectives will be particularly aided by their apparent reproducibility. Finally, the present results raise the possibility that such antisera may be useful for the detection of pheochromocytomas and related differentiated tumors such as neuroblastomas.

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