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New Cell Surface Antigens in Rat Defined by Tumors of the Nervous System

(glial and neuronal cell lines/ethylnitrosourea-induced tumors/embryonic antigens/brain antigens)

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ABSTRACT **Tumors of the central and peripheral nervous system were induced in rats with ethylnitrosourea. Many of these tumors were transplanted in syngeneic recipients, and several cell lines were derived from them. An antiserum raised against one such cell line in C_3H mice defined two cell surface antigens in cytotoxicity tests. One, the common antigen, was present on rat brain and embryonic tissues and was present in large amounts on most tumors or cell lines from the nervous system. Fibroblastic cell lines had smaller amounts of this antigen, which also could be detected by immunofluorescence. The other, restricted antigen was not detected on normal or embryonic tissues. It was present on six tumors from the nervous system, on one glial cell line, and on a Schwann-cell line RN22. In addition, it was present on four out of eleven cloned cell lines isolated from rat tumors at the Salk Institute. Two of the positive clonal lines had been shown to have properties unique to neuronal cells. The restricted antigen was therefore expressed on the cell surface of some, but not all, glial, Schwann, and neuronal neoplastic cells.**

The study of neural* cells in tissue culture provides a new approach to questions of the functions and interactions of the different populations of cells in the nervous system. However, physical methods for separating the different cell types have severe limitations, and identification of cells that have been treated with enzymes or grown in tissue culture is time-consuming and difficult. The use of surface antigens for distinguishing, separating, and studying the function of different types of lymphocytes (1) has suggested a possible immunological approach to the problems of neural cell populations. A number of murine allo-antigens (Thy-1, PC1, Sk, and H-2) are known to be present in mouse brain; if any one of them were restricted to a subset of neural cells, it might be a useful marker (2). In addition, new cell surface markers have been defined by using tumors of the nervous system. Recently an antigen on brain and murine neuroblastoma Cl300 (3), and a glial-specific brain antigen carried by oligodendroglioma G26 have been reported. The restricted distribution of the glial-specific antigen was determined by using five more tumors of nervous system, and several leukemias and sarcomas, but its distribution on the cell types in normal brain has not yet been determined directly (4). A third cell surface antigen has been defined by taking advantage of the clones of neuroblastoma Cl300 and their capacity for morphological differentiation in tissue culture, to reveal an antigen shared by brain and only the differentiated cells *in vitro* (5, 6). Again, the antigen-bearing cell type in normal brain, and the possible restriction of the antigen to neurons, remain open questions.

* The term neural is used throughout simply as meaning "from the nervous system."

While the murine tumors are an attractive system, it has proved difficult (ref. 7; Fields, unpublished) to induce more nervous system tumors in mice, so that only a few are available. In contrast, neural tumors can be induced in the rat with remarkably high efficiency and specificity by use of a carcinogen, ethylnitrosourea (8). While the majority of these tumors are Schwannomas or gliomas (8), some neuroblastomas have been reported (9), and the isolation of cell lines from these tumors has made available a well-characterized astrocytoma (10), a Schwannoma (11), and most recently a large collection of enzymatically and electrophysiologically characterized lines, including at least five neuronal clones (12). The large number and diversity of these rat tumors makes them particularly suitable for defining neural cell surface markers.

We have induced and transplanted a number of neural tumors in inbred rats, and developed several homogeneous cell lines from them. After many unsuccessful attempts, we have succeeded in producing an antiserum that defined two different surface antigens, one common to most of the tumors and to brain, the other restricted to a subset of the tumors.

MATERIALS AND METHODS

Tumors. *N*-Ethyl-*N*-nitrosourea (Schuchardt, Munich) was injected subcutaneously (50 mg/kg) into newborn, inbred Wistar/Furth (W/Fu) rats. Tumors arose in more than 90% of the animals 6–13 months later, and were in the brain (27%), cord (35%), lumbar cord and roots (18%), and spinal roots (14%) essentially as described (8, 9), except that we observed few tumors of the cranial nerves. The tumors were removed when the animal showed neurological symptoms, and a portion was fixed for histology. The remainder was finely chopped, injected subcutaneously into newborns, and put into tissue culture whenever possible. Many (72%; 29/40) transplanted, although the tumors of the cerebral hemispheres were notably slow to transplant in the first recipients (259 ± 24† days). Most tumors then transplanted regularly in 3–6 weeks, and were examined histologically and frozen at regular intervals. No tumor ever regressed or failed to transplant after the first two passages.

A W/Fu rat lymphoma (C58NT)D (13), induced by murine Gross leukemia virus and bearing abundant viral antigens, was maintained in ascites passage by Dr. G. Shellam of this department.

Cell Lines. All cells were grown in plastic flasks or dishes in Dulbecco's modified Eagle's medium, with 10% v/v fetal calf serum. This tissue culture medium was usually sup-

† All errors are expressed as ± SEM.

plemented with tryptose phosphate (1.2 g/liter) and antibiotics.

Several cell lines were developed from the tumors. Line 33B was derived from an ethylnitrosourea-induced tumor (TR33B) at its 17th transplantation passage. The original tumor involved both lumbar spinal cord and roots, and in the cord appeared to be an oligodendroglioma. In culture the cells grow to high density, form a sheet of cells with many processes, and morphologically resemble the Schwann cell line RN22 (11). Cell line 21A was derived from a transplantable tumor (TR21A) at its 6th passage *in vivo*. The original tumor was in the cerebral hemispheres. The transplanted tumor was not fibroblastic, but the cell line forms elongated, parallel, swirling arrays of cells in crowded cultures. Mouse cell line TR6B was derived from a tumor that originated in the trigeminal nerve and brain of a C3H/He mouse injected with ethylnitrosourea (230 mg/kg) intracranially on the day of birth (Fields, unpublished). This tumor transplanted readily and resembled a Schwannoma histologically; the cell line was derived from the first passage *in vivo*. The cultured cells form prominent processes, resemble 33B morphologically, and, unlike the rat cell lines, contain abundant C-type particles.

Established Cell Lines. Eleven clonal cell lines (B11, etc.; see Table 3) were a gift of Dr. D. Schubert of the Salk Institute. They were derived from ethylnitrosourea-induced brain tumors of BD-IX rats, and have been characterized by electrophysiological and biochemical tests as neuronal or nonneuronal (12). Two other cloned cell lines from alkylnitrosourea-induced tumors were used. C6 is an astrocytoma cell line (10) obtained from the American Type Culture Collection through Biocult (Glasgow), and from Dr. S. Pfeiffer, who also sent us RN22, a Schwann-cell line derived from a BD-IX rat nerve-root tumor (11). Other cells used were REL, a rat cell line with contact-inhibited, fibroblastic morphology, the gift of Dr. R. Bomford, and RE cells, cultured W/Fu embryo cells used between passages 4 and 12 after primary dissociation. XC, a Rous sarcoma virus-transformed rat fibroblast line (14) and an SV-40 virus-transformed mouse fibroblast line were obtained from Dr. N. Hogg. NS20Y (15), a clone of mouse neuroblastoma Cl300, was the gift of Dr. M. Nirenberg.

Antiserum. C3H/He mice were injected intraperitoneally at weekly intervals with 2×10^6 cells removed from plates with 0.5 mM EDTA in phosphate-buffered saline (EDTA/phosphate/saline) and washed several times. For the sixth and final injection, the cells were removed with trypsin (0.025% in EDTA/phosphate/saline), and given with 2×10^6 cells of sarcoma 37 to induce ascitic fluid, which was collected 8 days later. After centrifugation, the supernatant was stored at −20°, and heat-inactivated (56°, 30 min) before use. The antisera from three mice were indistinguishable, and for most experiments have been pooled.

Adsorption. All adsorptions were done at room temperature for 30 min with mixing. Normal tissues were cut up, sieved, and washed at least three times in phosphate/saline or tissue culture medium. Chopped tissue from tumor transplants was stored in 10% dimethylsulfoxide above liquid nitrogen, thawed, and processed similarly. Tissue culture cells were usually removed from plates with trypsin and EDTA; whenever tested, EDTA/phosphate/saline alone gave identical results. Repeated liver adsorptions were done with an equal volume of neat antiserum and packed tissue. Spleen and thymus were pooled and adsorptions were done with two volumes of neat antiserum to one volume of packed tissue. Unless stated otherwise, all tissues were from W/Fu rats. Nonspecific losses were assessed by parallel adsorptions with mouse tissues. The standard treatment of the crude antiserum was adsorption three times with liver and four times with pooled spleen and thymus. Unless it is specifically stated (e.g., Fig. 1), all experiments reported here start with this adsorbed antiserum. Further small-scale adsorptions were done with equal volumes (10–50 μl) of antiserum, tissue culture medium, and packed tissue or cells. After incubation, an additional two volumes of medium were sometimes added to reduce nonspecific losses. The small plastic vial was then spun at 14,000 × *g* in a desk-top centrifuge for 4 min. The supernatant was used for further adsorption or frozen for later testing.

Cytotoxicity Tests. Antisera were tested by a two-stage dye exclusion cytotoxicity assay. Target cells were normally removed from plates with trypsin and EDTA, and washed three times. All dilutions and washing were done in tissue culture medium. Twenty-microliter volumes of diluted antiserum and of cells (at 4×10^6/ml) were incubated 30 min at 37° in a CO_2-gassed incubator, then washed with 0.3 ml at 5°. The cell pellet was resuspended in 20 μl of unadsorbed rabbit complement (diluted 1:5). After a second 30-min incubation at 37°, the cells were pelleted. In turn, each was resuspended in 0.9% NaCl containing 0.16% trypan blue, and roughly 200 cells were scored for viability. Rabbit complement was essential for the detection of the cytotoxic activity of these sera. Toxicity of the complement itself was usually less than 10% for tumor cells, but was much higher with rat embryo cells (50%) or with thymocytes (30–70%). The complement was not varied, because only rough estimates of residual killing of these cells by the adsorbed antiserum were needed.

Immunofluorescence Tests. Antisera were tested against target cells in suspension by indirect immunofluorescence assay using an immunoadsorbant purified rabbit antibody against mouse immunoglobulin conjugated to fluorescein and a Vickers M41 microscope equipped with incident illumination and modified as described (16). Antisera were used at dilution between 1:8 and 1:256, and the proportions of cells with distinct surface fluorescence were estimated by examination of 20–200 cells.

RESULTS

Antisera from mice immunized with rat tumor cell line 33B had high cytotoxic titers (1:4000) against all rat cells tested, tumor or normal, and much of the activity could be adsorbed by normal rat tissues (Fig. 1). After extensive adsorption with liver, spleen, and thymus, the serum was not cytotoxic for thymocytes and had a titer of less than 1:4 against normal, cultured rat embryo cells. This adsorbed serum, used in all further experiments, had a remaining cytotoxic titer of 1:128 for the 33B cells. Three further adsorptions with liver did not significantly lower the titer, whereas a single adsorption with 33B cells removed it all (Fig. 1).

The adsorbed serum killed 21A and 33B cells equally well. In contrast, adsorption with 21A cells had very little effect on the cytotoxic titer for 33B cells (Table 1). The simplest interpretation is that the antiserum contains antibodies directed against two antigens: one on both 21A and 33B cells, the other only on 33B cells. A preliminary screen of several cell lines indicated that each one resembled either 33B or 21A. Therefore,

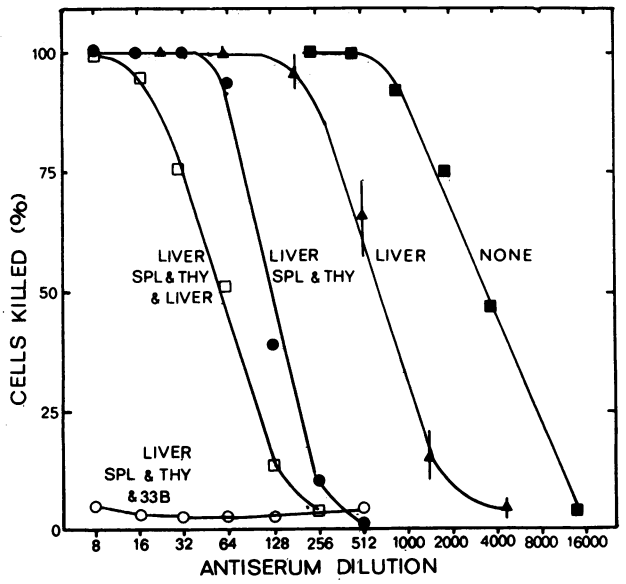

FIG. 1. Sequential adsorption by normal and 33B cells of the cytotoxic activity of anti-33B serum. Unadsorbed serum (■). The same serum adsorbed: 3× with liver (▲); 3× with liver, and 4× with spleen and thymus (●); 3× with liver, 4× with spleen and thymus, and 3× more with liver (□); 3× with liver, 4× with spleen and thymus, 1× with 33B cells *in vitro* (○). The target cell was the 33B cell line.

tissues and tumors were screened for antigenic content by measuring their ability to absorb cytotoxicity for these two cells. This analysis defined two antigens (or groups of antigens). The *common antigen*, detected by the use of 21A as the target cell, was shared by brain, embryonic tissue, and neural tumors. The *restricted antigen*, detected by the use of 33B as the target cell, was present only on a subset of neural tumors.

The common antigen

Adsorption tests with the cells used to define the antigens and with normal tissues (Table 1) show that the common antigen was present on both 21A and 33B cells, and on adult rat brain and peripheral nervous tissue (cauda equina), but it was absent from liver, spleen, thymus, and erythrocytes. Mouse brain was negative. The common antigen was not confined to nervous tissue, for whole rat embryo was as positive as brain. The sensitivity of the method was such that adsorption with as little as 3% of the standard amount of 33B cells was easily measured and reduced the titer by a factor of two. Rat kidney tissue had a very small amount of antigen that was detected only by multiple adsorptions. Bearing this in mind, it appears that the common antigen is reasonably specific for rat brain and embryonic tissue.

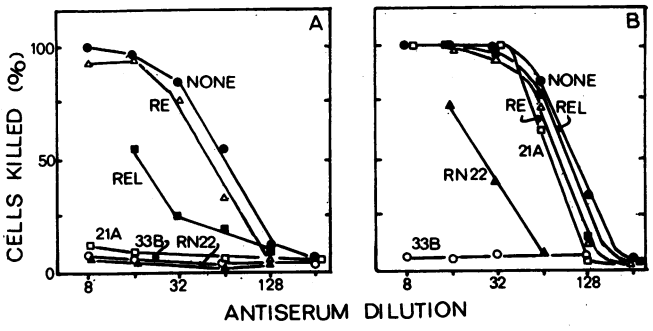

FIG. 2. Adsorption by *in vitro* cells of the cytotoxicity against two different cell lines. (A) Target cell was 21A; (B) target cell was 33B. The antiserum was adsorbed with liver, spleen, and thymus (●), and given one further adsorption with the following cells: RE, rat embryo cells *in vitro* (△); REL, rat embryo fibroblast cell line (■); or tumor cell line 21A (□), RN-22 (▲), or 33B (○).

TABLE 1. *Antigen distribution on defining cells and on normal tissues*

Adsorptions*	Common antigen Titer†	Common antigen Decrease‡	Common + restricted antigens Titer†	Common + restricted antigens Decrease‡
None	64	0	128	0
33B cells, 1×	<4	++++	<4	++++
21A cells, 1×	4	++++	64–128	±
Liver, 3×	32	(±)	64	(+)
Spleen and thymus, 1×	64	0	128	0
Erythrocytes, 2×	32	(±)	32	(+)
Kidney, 1×	32–64	(±)	128	0
Kidney, 3×	16	++	64	(+)
Brain, 1×	32	+	64–128	±
Brain, 2×	16	++	32–64	+±
Brain, 3×	<16	+++	32–64	+±
Mouse brain, 3×	32	(+)	64	(+)
Cauda equina, 1×	16–32	+±	64–128	(±)
Cauda equina, 3×			64	(+)
Embryo, 1×	16	++	64	+
21A plus one tissue§	8	++++	64	(+)

* Single (1×) or multiple (3×) adsorptions were done, starting with antiserum adsorbed in the standard way with liver, spleen, and thymus. Except for mouse brain, all tissues and cells were from W/Fu rats.

† The common antigen was measured with 21A as the target cell in cytotoxicity tests; the restricted plus the common antigen together, with 33B as the target. The titer is the antiserum dilution killing 50% of the cells.

‡ Each + represents a decrease in cytotoxic titer by a factor of 2; each ± by less than a factor of 2; 0 indicates no detectable decrease. Any decrease thought to be due to nonspecific losses is enclosed in parentheses.

§ The second adsorption was with liver, kidney, brain, cauda equina, or sciatic nerve.

Thirty ethylnitrosourea-induced transplanted rat tumors were screened for the antigens by adsorption tests; the results are summarized in Table 2. Most of the tumors (groups I and II) were highly positive for the common antigen, and such tumors originated in all parts of the nervous system. Four of the brain tumors in group II were passaged in tissue culture before producing transplantable tumors that were highly positive. The seven weakly or doubtfully positive tumors (group III) include two tumors that arose in the pituitary and may have no close relation to the nervous system tumors. The other tumors of group III originated within the nervous system, although none of these transplanted tumors shared the histologically glial appearance of TR33B or TR21A. C58, a Gross virus-induced lymphoma, was clearly negative. Thus, the common antigen was strongly expressed by most, but not all, ethylnitrosourea neural tumors.

Twenty-five cell lines were tested for their ability to adsorb the cytotoxic antibodies. Results shown in Fig. 2A illustrate the variation in the amount of the common antigen found on these cells. An adsorption with rat embryo cells *in vitro* did

TABLE 2. *Antigen content of transplanting tumors*

Group	Total no. in group	Antigen content* Common	Common plus restricted	Sites of origin of tumors
I	6	++++(5) +++(1)	++++(2) +++(3) ++(1)	Lumbar cord and roots (4), cerebellum (1), and thoracic cord (1)
II	17	++++(9) +++(7) ++(1)	+(9) ±(5) 0(3)	Brain (10), brain stem (1), spinal cord (1) or root (1)
III	7	+±(3) +(3) ±(1)	±(3) 0(4)	Brain (3), spinal cord (1) or root (1), and pituitary (2)
IV	1	0(1)	0(1)	Lymphoma (C58NT) D

* Antigen content was scored by adsorption of cytotoxicity. For the common antigen, 21A cells were the targets; for the common plus restricted antigens, 33B cells were the targets. The decrease in titer (see Table 1) after a single adsorption is shown, followed in parentheses by the number of tumors in the group with that value.

not reduce the titer of the antiserum; their lack of the common antigen by this test agrees with the insensitivity of such cells as targets but does not indicate which tissues and cells of the rat embryo have the antigen *in vivo*. As with transplantable tumors, most of the ethylnitrosourea-induced tumor cell lines were highly positive. Three lines, 33B, RN22, and 21A, are shown in Fig. 2A, and the results with more cell lines are summarized in Table 3, group A. In addition, eleven well characterized cell lines from the Salk Institute have been tested (Table 3, group C); all were highly positive. Three cell lines had intermediate amounts of common antigen: REL, a nontransformed, rat embryo fibroblast line; XC, a rat virus-transformed tumor cell line; and TR22, a tumor line from one of our nervous system tumors. The amount of antigen on these cells was about 6% as much as on 33B cells. Three mouse cell lines (a neuroblastoma clone, a Schwannoma, and simian virus 40-transformed fibroblasts) were all negative.

The restricted antigen

Most tissues were screened for the restricted antigen by adsorbing the standard antiserum and testing for toxicity against 33B cells (Table 1). Antibodies directed against the restricted antigens contribute nearly all the killing, as shown by the negligible effect of adsorption with 21A cells. No normal tissues showed definite adsorption. A second test, more specific for the restricted antigens, was carried out with some tissues. The antiserum was preadsorbed with 21A cells to leave only those antibodies directed against the restricted antigens, and then a second adsorption was done with different normal tissues. Liver, kidney, brain, cauda equina, and sciatic nerve were all negative in this test (Table 1, last line). It is important to note that neither brain nor embryonic tissues had clearly detectable amounts of the restricted antigen, whereas they both had small amounts of the common antigen.

Adsorption with *in vivo* tumor material showed that the restricted antigen was not unique to the cell line 33B. As

TABLE 3. *Antigen distribution on cells in vitro*

Group	Cell line	Cell type*	Antigen† Common	Common + restricted
A	33B	Glial	++++	++++
	RN22	Schwannoma	++++	++
	21A	Fibroblastic	++++	±
	TRV28B, TR17, 29A	Glial	++++	0
	TR 22	Transformed morphology	++	0
	C6	Astrocytoma	++++	±
B	RE	Embryo cells	0	0
	REL	Embryo cell line	++	0
	XC	Transformed fibroblast	++	0
C	B35, B103	Neuronal	++++	++++
	B50, B65, B104	Neuronal	++++	±
	B82	Non-neuronal	++++	++++
	B92	Non-neuronal	++++	++
	B11, B12, B15, B108	Non-neuronal	++++	±
D	TR6B	Mouse Schwannoma	0	0
	NS20Y	Mouse neuroblastoma	0	0
	SV-3T3	Mouse transformed fibroblast	0	0

* Cell lines in group A were derived from ethylnitrosourea-induced tumors, and the general morphology is indicated. Group C lines were from the Salk Institute. The cell type is that assigned in ref 12. Neuronal cells were electrically active (12).

† Antigen content was scored by adsorption of cytotoxicity. The decrease in cytotoxic titer is indicated. Target cells and scoring system are exactly as described in Table 1. A single adsorption with each cell type was performed.

shown in Table 2, it was found on the *tumor* TR33B and on the five other tumors of group I. Most of the ethylnitrosourea-induced tumors, those in groups II and III, had very low or undetectable amounts of the restricted antigen. Most of the antigen-positive tumors of group I originated in the lumbar cord and roots. The positive tumors transplanted rapidly in the first recipients (51 ± 18 days), if compared to the tumors of the cerebral hemispheres, which transplanted very slowly (259 ± 24 days). The presence of the restricted antigen on strictly *in vivo* tumors showed that it is not an antigen acquired by the 33B cells in tissue culture.

A number of cell lines were tested for the restricted antigen (Fig. 2B and Table 3). Of the six cell lines derived from our own ethylnitrosourea-induced tumors, only 33B was positive. C6 astrocytoma cells were negative, but RN-22, a Schwann-cell line from a spinal nerve root tumor (11), was positive. The possibility that the restricted antigen was only on Schwann-cell tumors or cell lines was tested by examining eleven cell lines from the Salk Institute (Table 3, group C). Three lines were highly positive, B35, B103, and B82. Two of these are electrically excitable, neuronal lines; one is not excitable and is of putative glial origin (12). One more non-neuronal line, B92, was also positive, but had less antigen.

The other seven lines, including electrically active and inactive clones, were negative. The restricted antigen, therefore, seems to cut across the usual divisions of peripheral versus central nervous system, or neuronal versus glial cells.

The possibility that the antigen is related to a virus is difficult to exclude, but several cell lines known to have viral origins or visible virus particles were tested. C58 (Gross virus), TR6B (C-type particles), NS20Y (possible A-type particles), XC (Rous sarcoma virus), and SV-3T3 (simian virus 40) were all negative.

Detection by Fluorescence. Both 33B and 21A cells showed strong surface staining in indirect immunofluorescence tests. The titer of the adsorbed antiserum was comparable to its cytotoxic titer. This immunoglobulin binding corresponded to the common antigenic system, for it was fully adsorbed by 21A or 33B cells, but not by rat embryo cells *in vitro*. Very little fluorescence of 33B cells could be seen after adsorption with 21A, indicating that the restricted antigen is not easily detected by this method.

DISCUSSION

Several immunological markers for the nervous system have now been described. S-100, a cytoplasmic protein, has been used as a measure of the degree of differentiation of cells (17) and tumors (11), but may not be useful for classifying clonal lines as neuronal or non-neuronal (12). Neither of the cell surface antigens on neuroblastoma Cl300 (3, 5) has been shown to be only on neuronal cells. The glial specificity of the antigen on oligodendroglioma G26 (4) depends on the results with a few mouse tumors. Our results with rat tumor cell lines indicate that it may be misleading to generalize from a single tumor to other similar tumors, or to normal cells of that cell type. Fortunately, the glioma G26 antigen (4) is also in the rat brain, so that all tumors of the rat system may be used to investigate the specificity of this antigen for glial or neuronal cells.

The presence of our common antigen on embryonic tissues *in vivo* raises some doubt as to its usefulness as a brain-specific marker. Tumors are known to express embryonic antigens, but these are not usually believed to be on adult tissues. Embryonic antigens are often defined by weak sera obtained from multiparous females or produced by the injection of embryonic tissue into the adult host. Each individual positive serum shows reactivity with a fairly small proportion of the tumors tested (18). In contrast, our serum reacts both with embryonic tissues and with adult brain, and very strongly with a striking majority of our ethylnitrosourea-induced tumors. It seems to define an antigen that is expressed widely in embryonic tissues, in a particular tissue (brain) in the adult, and most strongly by the majority of neural tumors. Antigens specific for human astrocytoma cells have been reported (19, 20), but neither of these antigens was found in normal adult brain.

It is not clear that the restricted antigen defined by our work can be directly compared with other tumor-associated antigens, which are usually defined by tumor rejection reactions of the syngeneic host. Many such reactions against chemically induced tumors are specific for the individual tumor. In contrast, our restricted antigen is associated with at least eleven independent tumors or cell lines. Where cross-reactivity is found, in some guinea pig tumors and in bladder carcinomas of several species, the possibility of crossreacting viruses or organ-specific antigens is often discussed (21). The presence of the restricted antigen on tumor material *in vivo* implies that any viral cause could not simply be an accident of tissue culture. Some of the ethylnitrosourea-induced tumors have been examined by electron microscopy and appeared free of viral particles (Fields and Fox, unpublished). Other cells with known viral origins lack the antigen.

The restricted antigen separates the various tumor cells into two classes, but the biological significance of this division is unknown. If the identifications of RN-22 as a Schwann cell, most of our tumors as gliomas, and the cell lines from the Salk Institute as both neuronal and non-neuronal cells are all correct, and if the restricted antigen is ever expressed by normal cells, then it may be shared by both the peripheral and central nervous system, and by neuronal and non-neuronal cells.

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