

NS-1 (Nervous System Antigen-1), a Glial-Cell-Specific Antigenic Component of the Surface Membrane

(murine oligodendroglioma G26/glia-cell-surface-specific antigen/myelin and myelin synthesis/crossreactivity among mammalian species)

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Communicated by Francis O. Schmitt, January 31, 1974

ABSTRACT A methylcholanthrene-induced glioblastoma of the C57BL/6 inbred mouse strain was used to raise antibodies in C57BL/6 and C57BL/10 inbred mice and in (C57BL/6 × DBA/2) and (C57BL/6 × Balb/c) F₁ hybrids. When examined by the cytotoxicity test, these antibodies define a cell-surface component (or components) found exclusively on brain tissue of all mouse strains studied and of several other mammalian species including man. The antigen, named NS-1 (nervous system antigen-1), is present on cells of three of the four mouse-glia-cell tumors tested, but not on the C1300 neuroblastoma, a tumor of neuronal origin. NS-1 occurs in higher concentration in regions of the nervous system richer in white than in gray matter, and in lower than normal concentrations in brains of myelin-deficient neurological mutant mice. The concentration of NS-1 gradually increases postnatally and reaches the adult level between the third and fourth week. The existence of more than one allele or genetic locus controlling NS-1 activity is suggested by the occurrence of higher amounts of NS-1 in brains of the A and C57BL/6 than of the Balb/c and DBA/2 mouse strains. NS-1 is the first cell-surface component to be described that is not only unique to nervous tissue, but specific for glial cells.

The mammalian brain is composed of diverse cell populations with different functions. In order to gain insight into these functions at the cellular and molecular levels, individual cells and, as a first approximation, particular classes of cell types must be characterized. An intimately related aim is the separation of subpopulations of nerve cells.

The cell surface is of particular interest in these respects, since it may play an important role in the social behavior of cells during morphogenesis and differentiation (ref. 1 for review, and refs. 2 and 3), and since separation of cells can be achieved with the help of reagents that recognize surface differences [i.e., antibodies, lectins (4-6)]. The present study approaches these objectives by exploiting the sensitive and discriminatory capacity of antibodies to define a surface marker which is specific for one particular cell type.

A major difficulty in delineating antigenic surface components that distinguish among classes of brain cells is that the cell populations available as sources of antigen are far from pure (7-11). One is caught in a vicious circle since very pure cell populations are needed as antigens in order to raise specific antisera, while at the same time the immunological approach itself is expected in time to provide the desired separations.

We have turned to the use of tumors as sources of antigen because they may retain some properties characteristic of normal cells (12-17), because they grow in relatively homogeneous form, and because the cells are available in preparative amounts. The present report utilizes a chemically induced brain tumor of the mouse, the glioma 26 (18-20), to raise antibodies in mice that characterize a cell-surface component (or components) that is specific for a glial subpopulation of nervous system cells.

MATERIALS AND METHODS

Mice. All inbred strains used in this study were obtained from the breeding colonies of the Memorial Sloan Kettering Cancer Center, New York, N.Y., the Department of Neuropathology, Harvard Medical School, Boston, Mass., and The Jackson Laboratory, Bar Harbor, Me.

Tumor. Glioma 26 was induced with methylcholanthrene in the C57BL/6 inbred mouse strain (abbreviated B/6) by Dr. H. M. Zimmerman 30 years ago (20) and was obtained from Drs. K. Sugiura and W. R. Shapiro (Memorial Sloan Kettering Cancer Center) (18, 19). It was classified as an oligodendroglioma in the primary tumor and did not dedifferentiate upon serial subcutaneous passage. The tumor grows *in vivo* as a morphologically homogeneous solid mass (19). It carries antigenic cell surface markers that are found in normal mouse brain and biochemical markers characteristic of glial cells (unpublished results). The tumor was maintained in B/6J males by subcutaneous or intraperitoneal passage at intervals of 2-3 weeks. When a suspension of tumor cells (between 1×10^6 and 1×10^7) was injected into the peritoneal cavity, nodules of tumor formed on the peritoneal lining. The percentage of viable cells was higher in peritoneal than in subcutaneous masses.

Antisera. Anti-glioma G26 antibodies were raised in female mice of the following strains: B/6J, B/10J, (B/6 × DBA/2) F₁, and (B/6 × Balb/c)F₁ hybrids. Cells for immunization were prepared by mechanical dispersion of the *in-vivo*-grown solid tumor mass, washing three times in Earle's balanced salt solution (EBSS) and irradiating with a cobalt source at a dose of 15,000 rads for the first four immunizations (approximately 1×10^7 cells per animal without adjuvant; 10-20% of the injected cells excluded trypan blue) and 10,000 rads for later immunizations. Mice were bled starting from the ninth immunization at 6-8 days after the last boost. Sera used in the present studies came from different bleedings of individual mice with different antibody titers.

Abbreviations: B/6, mouse strain C57BL/6; B/10, strain C57BL/10; EBSS, Earle's balanced salt solution; G26, glioma 26; NS-1, nervous system antigen-1.

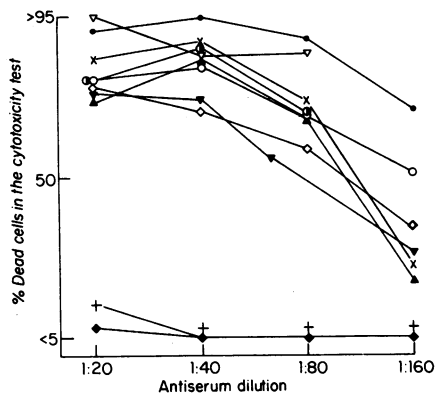


FIG. 1. Representation of NS-1 on various mouse tissues as determined by absorption of cytotoxic antibody. ●, unabsorbed antiserum (B/6 \times DBA/2) F₁ anti-G26; antiserum absorbed with: ×, B/6 liver; ○, K36 spontaneous leukemia of (B/6 \times AKR)F₁ hybrid; +, B/6 brain; ◆, glioblastoma G26; ◇, B/6 spleen; ▲, B/6 thymocytes; ▼, B/6 kidney; ⊙, B/6 epidermal cells; ▽, B/6 testicular cells.

Serological Tests. Cytotoxicity test and absorptions were carried out as described previously (21, 22). Briefly, for the cytotoxicity test, tumor cells were mechanically dispersed, washed once in EBSS, subjected to trypsin and DNase treatment (0.25% and 0.025%, respectively, from Worthington) for 7 min at 37° at a concentration of approximately 5×10^7 cells per ml and washed three times with EBSS containing 10% immune precipitated fetal-bovine serum (Gibco). In the cytotoxicity test, tumor cells (2×10^6 cells per ml) were incubated with antiserum dilutions in a volume of 0.05 ml for 30 min at room temperature, washed once with 1 ml of test medium (Medium 199 containing 2% of immune precipitated fetal-bovine serum) and incubated for 30 min at 37° with 0.05 ml of rabbit complement (diluted 1:12) which was selected for low toxicity and high complement activity. The

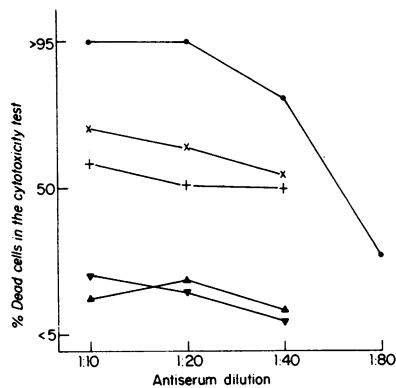


FIG. 2. Distribution of NS-1 on adult brains (more than 3 months old) of four mouse strains (A, B/6, Balb/c, and DBA/2) as determined by quantitative absorption. Absorption was carried out with various ratios of antiserum volume to packed tissue homogenate volume. In this case the ratio was 2:1. Similar curves were obtained by using ratios of 3:1 and 4:1, the difference between group B/6 and A and group Balb/c and DBA/2 being more pronounced. At a ratio of 1:1 all cytotoxic activity was removed by brains of all four strains at an antiserum concentration of 1:10, (B/6 \times DBA/2) anti-G26. ●, unabsorbed antiserum; antiserum absorbed with brain tissue of strains: ▼, B/6; ▲, A; +, DBA/2; ×, Balb/c.

activity of the antiserum was calculated by determining the percentage of dead cells (as judged by trypan blue uptake, 0.16% in 0.9% saline) in the total cell suspension. For absorption, antiserum at a concentration of two to four serial dilutions below the titer endpoint was incubated for 30 min at 4° with tissue homogenate that had been washed three times and contained mostly membrane fragments and a few whole cells without processes. The tissue homogenates were not subjected to enzymatic treatment. The ratio of serum to packed tissue homogenate was 1:1. The activity of the absorbed serum was tested in the cytotoxicity test as outlined above and described in further detail in ref. 22. Indirect immunofluorescence was carried out (23) with antiserum at various dilutions in medium containing 2% immune-precipitated fetal-bovine serum (Gibco). 1×10^6 trypsinized G26 tumor cells were incubated in 0.05-ml volumes of the diluted antiserum for 30 min at 4°, washed twice, and incubated with fluoresceinated rabbit anti-mouse Ig (Hyland Laboratories) at a dilution of 1:10 for another 30 min at 4°. After two washings cells were resuspended in three drops of 50% glycerol in phosphate-buffered saline, pH 7, and examined with a Leitz Orthoplan microscope equipped with an Osram HBO 200W mercury lamp, and BG38 excitor, KP490 interference and K530 barrier filters.

RESULTS

When B/6, B/10, (B/6 \times DBA/2)F₁, and (B/6 \times Balb/c)F₁ hybrids are inoculated with the B/6-derived glioma 26, an antigen, NS-1, is recognized that is unique to brain tissue (Fig. 1). No other normal mouse cells, including the ectodermally derived testicular and epidermal cells, and none of several malignant mouse cell lines absorb cytotoxic activity of anti-NS-1 antiserum using G26 as the indicator cell. The tumors studied, in addition to the leukemia described in Fig. 1, were Balb/c ascites plasmocytoma MOPC70A, B/6 ascites leukemia EL4, A/J sarcoma AI, A strain spontaneous ascites leukemia ASLI, Balb/c ascites sarcoma MethA, and A strain radiation-induced leukemia RADAI. Anti-NS-1 antibody falls into the category of autoantibodies, since the mouse strain that carries antibodies against the tumor also produces antibodies against a cell-surface constituent of its own brain. None of the hyperimmune animals show symptoms of neurological disorder by behavioral criteria. This may be due to the inaccessibility of NS-1 to peripherally circulating antibodies or killer lymphocytes because of the blood-brain barrier. By contrast, injection of basic encephalitogenic protein together with complete Freund's adjuvant can induce autoimmune experimental allergic encephalomyelitis (24).

Brain tissue from all mouse strains tested (B/6, B10J, DBA/2, Balb/c, C3H/Bi, AKR, and 129) carries NS-1. When brains from these strains are assayed for relative concentration of NS-1 (amount of antigen per volume of packed tissue homogenate as measured by absorptive capacity) there are quantitative differences. Strains B/6 and A show about three times higher absorbing capacity for antibody than strains Balb/c and DBA/2 (Fig. 2). This experiment was performed six times with consistent results.

In order to ascertain the cell types in the nervous system that express NS-1, the following three experiments were performed: (a) determination of NS-1 concentration in a brain region enriched in white matter (tissue taken from the brainstem) and in a brain region enriched in gray matter (tissue taken from the isocortical mantle of the cerebrum,

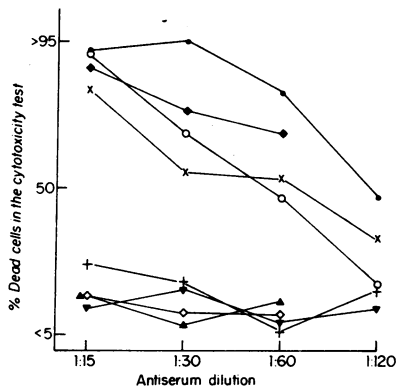


FIG. 3. Distribution of NS-1 on white and gray matter and on four tumors of glial and one of neuronal cell origin as determined by absorption. ●, unabsorbed antiserum (B/6 × DBA/2) F₁ anti-G26; antiserum absorbed with: +, B/6 white matter from the brainstem region; ×, B/6 gray matter from the cerebral cortex; ▼, B/6 glioblastoma G26; ○, B/6 glioblastoma Sato; ▲, B/6 glioblastoma G261; ◇, B/6 ependymblastoma EPA; ◆, A neuroblastoma C1300.

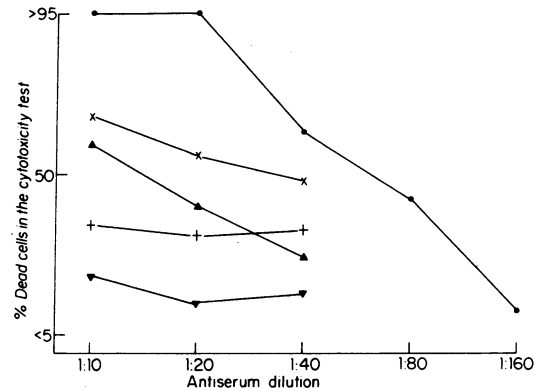


FIG. 4. Representation of NS-1 in brains of two myelin-synthesis-deficient mouse mutants (*msd* and quaking) and their normal littermates as determined by absorption. ●, unabsorbed antiserum (B/6 × DBA/2)F₁ anti-G26; antiserum absorbed with brain tissue from: ×, *msd* male, 24 days old; +, *msd* female (normal control littermate); ▲, quaking (*qk/qk*), 52 days old; ▼, -/+ (normal control littermate of quaking).

which contains little underlying white matter) (Fig. 3); (b) comparison of NS-1 representation in brains of two myelin-deficient mutant mice (the sex-linked mutation myelin synthesis deficiency, *msd*, and the autosomal mutation quaking, *qk*,) with their normal littermates (Fig. 4); and (c) determination of NS-1 content on tumors of neuronal and glial cell origin. Whereas only one neuronal cell tumor [neuroblastoma C1300 (12, 25)] is at present available in the mouse, three chemically induced glial cell tumors of the B/6 mouse strain could be examined [glioblastoma (14), glioma 261 (19), and ependymblastoma EPA (19)] (Fig. 3).

The higher concentration of NS-1 in white than gray matter and the reduced titers in myelin-deficient mutants compared to their littermates suggest that NS-1 may be associated predominantly with glial membranes. Glial-cell-rich regions of normal peripheral nerve taken from the trigeminal nerve trunk also express levels of NS-1 similar to those of white-matter-rich regions of the central nervous system (not shown in figures). Also, three of four glial cell tumors tested carry the antigen, whereas the C1300 neuroblastoma, a tumor of peripheral neuronal origin, does not express it. From the above experiments it cannot be determined, however, which glial-cell types carry NS-1 predominantly, though the oligodendroglial and Schwann cells responsible for myelination are likely among them. The fact that the four glioblastomas examined have been classified into different morphological categories (18-20) points to the possibility that NS-1 is expressed on more than one glial-cell type in the central nervous system.

Since the conclusion that NS-1 is a glial-cell-specific antigen is based on absorption of antibody with subsequent titration of its residual complement-dependent cytotoxicity, the question must be asked whether the apparent higher absorptive capacity of white matter as compared to gray matter is real or might reflect a stronger anticomplementary effect of the antiserum that had been in contact with white matter during absorption. Three points argue against this possibility:

(a) The titers of antisera against cell surface antigens like H-2 (22), Thy-1 (θ) (25), and Pc (21) are not reduced by anticomplementary effects of washed brain homogenates as shown with brains from the negative allogeneic mouse strain.

(b) In the cytotoxicity test as performed in these studies the target cells were washed after the antibody had attached and before the complement was added.

(c) The immunofluorescence method does not depend on complement, and gives similar results to those obtained by the cytotoxicity test.

Since myelination is a prominent feature of postnatal glial-cell differentiation, and since glial-cell proliferation continues into the first few weeks after birth (26, 27), it was of interest to determine NS-1 concentrations at various postnatal stages. The antigen is just detectable by the absorption technique on postnatal day zero and reaches the adult level at approximately 4 weeks (Fig. 5). One cannot say, however, whether this accretion signifies a quantitative increase in glial-surface membranes or a qualitative maturation of already generated cell-surface components, nor can one assess the possible contribution of the many other developmental events preceding during this time period.

The occurrence of NS-1 within different animal species was examined. Fig. 6 shows that NS-1 activity can be detected in all four mammalian species examined (mouse, rat, cat, and human). Partial crossreactivity of the human NS-1 equivalent with the antigen of the mouse was determined by ab-

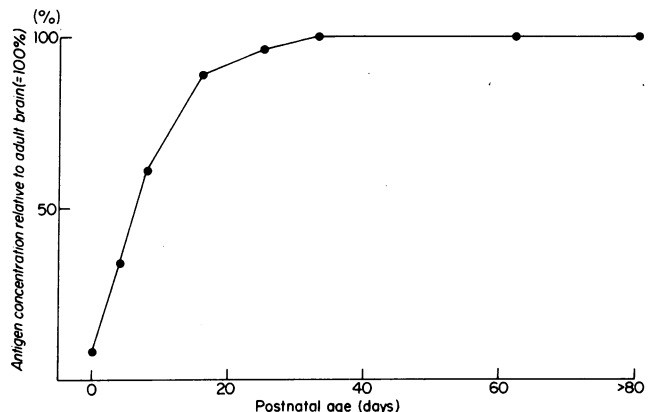


FIG. 5. Expression of NS-1 on whole mouse brain as a function of postnatal age.

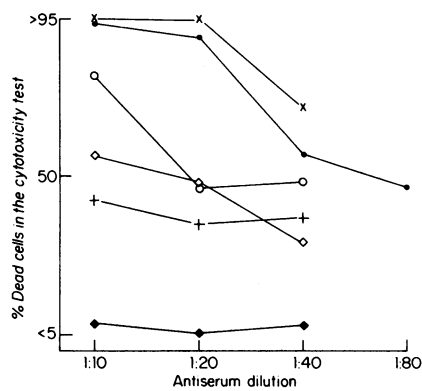


Fig. 6. Representation of NS-1 on brains of several animal species as determined by absorption. ●, unabsorbed antiserum (B/6 × DBA/2) F₁ anti-G26; antiserum absorbed with brain tissue from: ◆, mouse; +, rat; ◇, cat; ○, human; ×, chicken.

sorbing anti-NS-1 antiserum with frontal lobe of human cerebral cortex, containing a mixture of gray and white matter. One aliquot of this antiserum was titrated for residual cytotoxic antibody. Two other aliquots were further absorbed, one with mouse and the other with human cortical brain tissue. Whereas, the absorption with human brain did not further reduce the activity, absorption with mouse brain did reduce it to the background level. It can be concluded, therefore, that the human analog of NS-1 does not contain all antigenic specificities that are carried by the antigen from the mouse. Crossreactivity for the antigenic specificities in rat and cat brain were not determined, but it may be inferred by extrapolating from the above experiment that the value for absorptive capacity of rat and cat brains represent crossreactivity and not differences in antigen concentration. Least crossreactivity is found with the phylogenetically most remote mammal, man, whereas, chicken shows no crossreactivity detectable by the absorption method. NS-1 thus adds to the list of brain proteins [S-100 (28), 14-3-2 (28), and basic protein of myelin (24)] that retain a considerable structural constancy during phylogeny.

DISCUSSION

NS-1, a glial-cell-specific antigen is the first specific cell-surface component to be described with an exclusive representation in the nervous system. Not only is it confined to this one organ, but it seems restricted to a particular class or few classes of cells. This puts it into the category of "differentiation" antigens (31). Apart from the Ly-series of alloantigens which are expressed only on thymocytes and thymus-derived lymphocytes of mice (29, 30), T1 alloantigen, which is expressed only on thymocytes (31), and the surface-membrane-bound immunoglobulins (ref. 32 for review), none of the hitherto recognized surface constituents that were defined with homologous (murine) antibodies meets this criterion of restricted tissue distribution.

Several tissue-restricted antigens have been recognized with hetero-antisera prepared in rabbits by immunization with mouse lymphoid cells. These sera were rendered specific for particular cell types by absorption of nonspecific anti-mouse species antibodies with other mouse tissues. These procedures have led to the identification of MSLA (33), MBLA (34), and MSPCA (35), all surface antigens of cells in the immune system. Efforts to produce a specific anti-G26

heteroantiserum in the rabbit have failed so far. Several nervous-system-specific cytoplasmic (probably not surface-membrane-bound) antigenic components have been reported: S-100 (28), 14-3-2 (28), antigen alpha (36), glial acidic fibrillary protein (37), a glycoprotein (38), and a protein unique to the olfactory bulb (39). Membrane-bound constituents of subcellular nerve-cell fractions, e.g., synaptosomes, have been detected with serological methods (40-43), but stringent evidence for a surface localization of these antigens was not sought. The methods of complement fixation with subcellular membrane preparations (40, 42, 43) and electrophysiologic recording from impaled nerve cells (41) used for detection of these antigens does not, in fact, allow for this distinction, in contrast to the cytotoxicity test used in the present study, which permits detection only of surface antigens.

The successful use of a brain tumor for characterization of a cell-surface antigen shared with normal brain cells justifies an intensive search for other nerve-cell surface markers. One might seek not only markers that distinguish among cell classes, but also among developmental stages of a particular cell class. The latter seems particularly plausible, since developmental cell-surface components are thought to be derepressed in some neoplastic cells. Homogeneity of cell type in the tumor is of great importance, and in this respect glioma G26 was a fortunate choice, since it grows *in vivo* as a solid tumor mass containing apparently only one major cell type with almost no contaminating connective tissue (ref. 19 and unpublished observations). The crossreactivity of NS-1 among mammalian species may open the possibility of using the more readily available brain tumors from rat (44) and man (15) for the definition of surface components shared by different species, though the inbred mouse offers the best prospects for recognition of allelic differences and for genetic mapping in mammals.

The author is indebted to Dr. R. L. Sidman for his comments on the manuscript. Part of this study was carried out at the Memorial Sloan Kettering Cancer Center and during the tenure of fellowships from the Deutsche Forschungsgemeinschaft and the Alfred P. Sloan Foundation and with the help of a grant from the National Science Foundation (GB-34186).

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