

## 4.4 Generation of monoclonal antibodies against human glioma associated antigens and their clinical relevance

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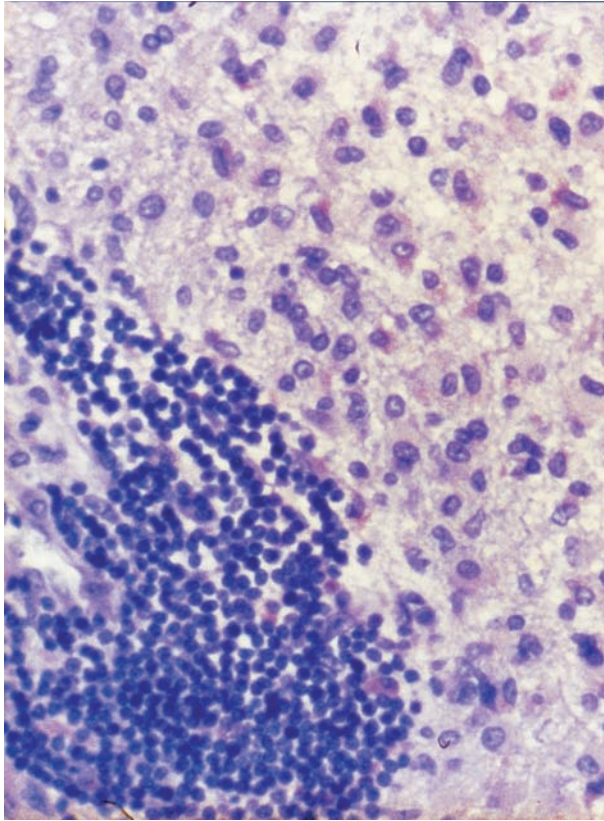
The presence of tumour associated antigens in human gliomas has been suspected since Reichner [1933] immunized dogs with boiled suspensions of glioma cells and normal brain tissue. He found that glioma extracts yields higher complement fixation titers in the resultant sera than normal brain extracts. This lead him to believe that gliomas have immunological characteristics not present in normal brains. Since that time, however, numerous experimental studies designed to demonstrate GAA have given conflicting results. This is, of course, not surprising particularly because inbred animals were not available before 1948, and the differences between immune responses against histocompatibility antigens and tumour associated antigens, were not appreciated.

Evidence for the possible existence of GAA has come from studies using in vitro microcytotoxicity techniques to demonstrate reactivity of peripheral lymphocytes to autogenous gliomas [Levy et al. 1972]. Additional, indication suggesting the immunogenicity of gliomas was obtained in studies concerning the cytotoxic effects of serum

from astrocytoma patients on cultivated autogenous and allogeneic cells [Kornblith et al. 1974]. Interesting experiences with rabbit antiglioma sera together with studies concerning the antigenicity of human gliomas are reported in the excellent reviews of brain tumour immunology [Mahaley 1978; Neuwelt and Clark 1978; de Tribolet and Carrel 1980; Dietrich 2001; Parajuli and Sloan 2004].

Further experimental studies aimed at demonstrating antibodies against chemically induced gliomas in syngeneic rats strongly suggested, that glioma cells express epitopes recognizable by the immune system [Stavrou et al. 1978, 1980]. The stimulus for this work came from earlier investigations in this laboratory which dealt with the presence and nature of lymphocytic infiltrates in gliomas (Figure 1). By means of specific antirat and anti-human T-lymphocyte immunoglobulin's and the fluorescent antibody fixation test a variable percentage of T-lymphocytes could be demonstrated among mononuclear cell infiltrates in human and experimental gliomas [Stavrou et al. 1977].

The studies concerning the polyclonal immune response to neurogenic tumours in several biological systems have collected enough evidence in support of the antigenicity of glioma cells



**Figure 1.** Demonstration of glioma associated antigens in a human astrocytoma III by means of MABs. Note evident perivascular lymphocytic infiltrates intermingled with antibody binding tumour cells.

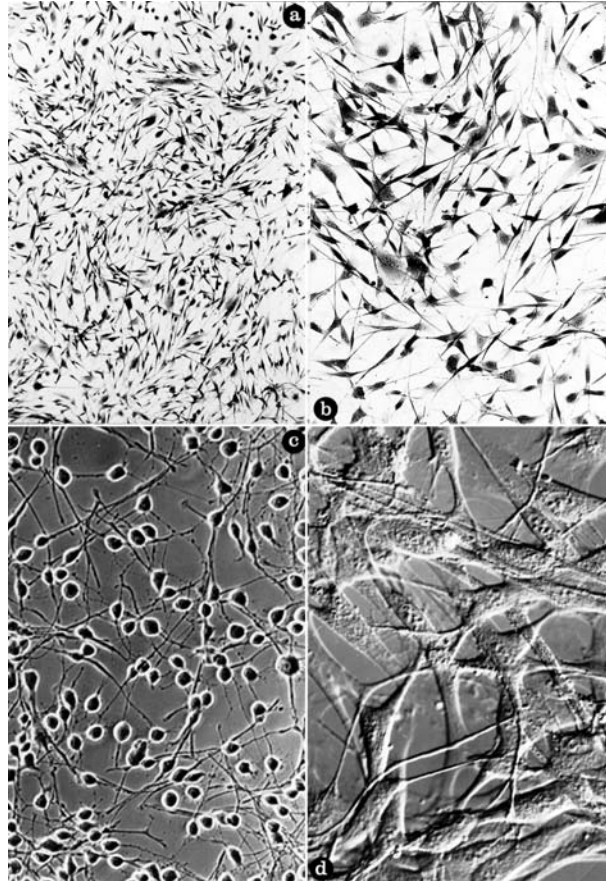
[Stavrou et al. 1980, 1982; Coakham et al. 1980, 1982]. However, the identification and characterization of glioma associated antigens by means of polyclonal sera was extremely difficult due to the large numbers of contaminating antibodies.

Despite the extensive efforts in order to isolate and describe GAA analysing polyclonal serological and cell mediated reactions in different systems such antigens could be archived in no instance.

The hybridoma technology applied to produce MABs of predefined specificities demonstrated not only the expression of tumour associated antigens (TAA) but also greatly stimulated the suitability of MABs in the rapidly changing field of oncological research.

In order to generate molecules useful to assist in the more accurate and objective typing of intracranial malignancies as well as to analyse the surface structures of glioma cells, hybridomas producing monospecific highly affinity immunoglobulins were established in several laboratories [Stavrou and Süß 1982, 1989; Epenetos 1991; Parney et al. 2000; Fecci and Samson 2002].

Immunoglobulin producing hybridomas arose from fusion of mouse myeloma cells (X63-Ag8.653) and spleen cells from female BALB/c mice hyperimmunized against human malignant astrocytomas were set up and propagated *in vitro*. In the following we describe briefly the different steps of the used fusion protocol and the recognition pattern of selected MABs with their



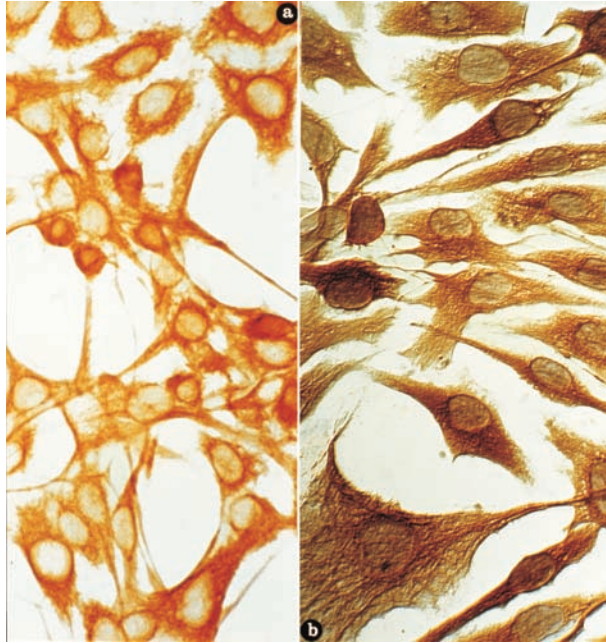
**Figure 2.** Uncloned human glioma cell culture (a,b) and cloned (c,d) permanent cell line (85 HG-63) used for fusion with the myeloma cell line X63-Ag8.653. (a-c: HE-stain; c: phase contrast; d: Nomarski interference contrast).

high degree of specificity utilizing cell cultures of different origin as well as tissue samples obtained from neurogenic and non neurogenic tumours and normal organs.

**Human gliomas:** Fresh tumour samples from biopsy material were immediately frozen and preserved in liquid nitrogen containers of bio-freezers until used for histochemistry or immunization. Additional tumour specimens were fixed in BOUIN's solution, KARNOVSKY's fixative and methanol/acetone mixture and embedded in paraffin (45 °C). Sections of frozen and paraffin material were used for conven-

tional histology as well as for immunohistochemistry. For control experiments tissue fragments of different regions of normal brain and other organs were processed analogous.

**Human glioma cell cultures:** For establishment of primary cultures, fragments of biopsy material obtained under sterile conditions were placed in Falcon flasks and covered with medium M 199, supplemented with 20% inactivated FBS, 10 ml 8,8% NaHCO<sub>3</sub>, 100,000 I.U. penicillin G, 0.1 g streptomycin, 0.1 g neomycin, and 3.4 ml glutamin (20 mM) per litre medium. Cultures were incubated at 37 °C in air with 6% CO<sub>2</sub>



**Figure 3.** Long term human glioma cell line (85 HG-63) used for MABs-gaining, consists of cells forming numerous extensions and showing a positive reaction for S-100- (a) and GFAP- (b) protein.

and high humidity. For in vitro passaging of the cells, primary cultures were splitted 1:2 using saline trypsin-versene (STV) solution. Long term cultures (Figure 2 and 3) were fed with RPMI 1640, supplemented with 10% inactivated FBS, 10 ml 8.8%  $\text{NaHCO}_3$ , 100,000 I.U. penicillin G, 0.1 g streptomycin, and 10 ml glutamin (20 mM) per litre medium, cloned (“cylinder cloning technique”) and karyotyped from time to time (Figure 4).

**Human embryonic cell cultures:**

Fragments from brain anlage as well as from thoracic and abdominal segments of two embryos (9<sup>th</sup> and 11<sup>th</sup> weeks of its development in the uterus) were layered in plastic flasks. All primary cultures were fed with HC medium (450 ml DMEM, 75 ml FBS, 50 ml NCTX-135 medium, 5 ml sodium pyruvate, 0.1 g neomycin) initially supplemented with 20% inactivated FBS and the subcultures were maintained in the same me-

dium containing 10% FBS. Subsequently, the cultures were serially transferred using STV solution twice a week. All cultures were incubated at 37 °C humidified atmosphere of 6%  $\text{CO}_2$  in air.

**Animals:** BALB/c and BALB/c-nu/nu mice used for immunization and production of ascitic fluid were taken from our barrier maintained colony.

**Mouse myeloma line:** The myeloma cell line X63-Ag8.653 [Kearney et al. 1979] was used for fusion experiments. The myeloma cells were grown in HC medium supplemented with 10% FBS. Two days before fusion, the cells were fed with HC medium containing 20% FBS.

**Establishment of murine hybridoma clones (MUC):** BALB/c mice were immunized i.p. three times (1<sup>st</sup>, 8<sup>th</sup>, 15<sup>th</sup> day) with  $5 \times 10^6$  TNBS (2, 4, 6 – trinitrobenzene sulfonic acid) modified whole human tumour cells culti-