An insight on the antigenic preparation strategies involved in the production of polyclonal antibodies against oligodendrogliomas (cerebral tumors).

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Abstract- Various strategies have been used to identify and characterize the antigens associated with human brain tumors. These approaches have included the raising of polyclonal and antibodies against tumor antigens and, more recently, efforts toward the direct biochemical identification of such proteins. This article summarizes the progress made in this area, suggests reasons for the broad antigenic cross-reactivity and heterogeneity revealed by these studies, and proposes additional methods such as radial and double immunodiffusion and ELISA for deciphering the complex antigenic composition of human brain tumors.

INTRODUCTION

Tumors are aberrant organ systems containing a complex interplay between the neoplastic compartment and recruited vascular, inflammatory, and stromal elements. A brain tumor is an intracranial solid neoplasm, a tumor (defined as an abnormal growth of cells) within the brain or the central spinal canal [1]. Brain tumors include all tumors inside the cranium or in the central spinal canal. They are created by an abnormal and uncontrolled cell division, normally either in the brain itself (neurons, glial cells (astrocytes, oligodendrocytes, ependymal cells, myelin-producing Schwann cells), lymphatic tissue, blood vessels), in the cranial nerves, in the brain envelopes (meninges), skull, pituitary and pineal gland, or spread from cancers primarily located in other organs (metastatic tumors) [2] . Any brain tumor is inherently serious and life-threatening because of its invasive and infiltrative character in the limited space of the intracranial cavity. However, brain tumors (even malignant ones) do not automatically cause death [3]. Brain tumors or intracranial neoplasms can be cancerous (malignant) or non-cancerous (benign); however, the definitions of malignant or benign neoplasms differs from those commonly used in other types of cancerous or non-cancerous neoplasms in the body [4]. Oligodendrogliomas are a type of glioma that are believed to originate from the oligodendrocytes of the brain or from a glial precursor cell. They occur primarily in adults (9.4% of all primary brain and central nervous system tumors) but are also found in children (4% of all primary brain tumors). The average age at diagnosis is 35 years [5]. Oligodendrogliomas arise in the cerebral hemispheres and are distributed among the frontal, parietal, temporal, and occipital lobe, in approximately a 3:2:2:1 ratio. Rarely, they can arise in the cerebellum, brain stem, and spinal cord. They usually occur in the cerebral white matter and are very cellular, with uniform nuclei. They react with glial fibrillary acidic protein on immunostaining [6]. In response to the cerebral tumor many different types of antibodies are produced, called the 'immunoglobulins'. In each case of glioma , only one type of immunoglobulin is made, but which will vary from patient to patient. There are 5 basic immunoglobulins – A, G, M, D and E. In oilgodendroglioma the commonest that is overproduced is Immunoglobulin G and low amount of Immunoglobulin M. Further there are two types of light chain, kappa and lamda on which the type of glioma depends. Oligodendrogliomas and mixed gliomas account for approximately 4 to 5% of all primary brain tumors and 10% of all gliomas. A primary brain tumor is a tumor that begins in the brain, as opposed to a secondary (or metastatic) brain tumor, which originates in another organ and spreads (metastasizes) to the brain [7].

This short project study was based on the evaluation of the polyclonal antibodies, particularly Immunoglobulin G, produced in response to the antigenic preparation of oligodendroglioma .Hence varies assays like SDS-PAGE, ELISA and double immunodiffusion were performed.

DEVELOPMENT AND CLASSIFICATION OF OLIGODENDROGLIOMA

It was recently demonstrated that the combined loss of 1p and 19q is mediated by an unbalanced translocation of 19p to 1q [8]. Most likely, a centrosomal or pericentrosomal translocation of chromosomes 1 and 19 results in two derivative chromosomes, der(1,19)(p10;q10) and der(1,19)(q10;p10), after which the derivative chromosome with the short arm of chromosome 1 and the long arm of chromosome 19 is lost.]. In tumors in which codeletion of 1p and 19q is present, the codeletion is generally distributed throughout the tumor, even in areas with more astrocytic morphology [9]. Furthermore, the loss of 1p and 19q is retained at the time of progression, regardless of morphological changes, suggesting that 1p/19q loss is an early genetic event [10].

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Oligodendrogliomas occur mainly in the cerebral hemispheres of adults. They are believed to derive from oligodendrocytes. They consist of moderately cellular, monomorphic tumours with round nuclei, often artefactually swollen cytoplasm on paraffin section (*Figure 1.1*), few or no mitoses, no florid microvascular proliferation or necrosis, and are classified as malignancy grade II according to the WHO. They do not express any antigen characteristic of normal oligodendrocytes. Grade II oligodendrogliomas are relatively indolent, although they usually reoccur at the primary site and may display a tendency for subependymal spread with a 5% incidence of cerebrospinal fluid (CSF) seeding. Oligoastrocytomas consist of tumour cells with either astroctytic or oligodendroglial morphological characteristics. Tumour cells with these two phenotypes can be either diffusely mixed or combined as discrete areas in an individual tumour (*Table 1.1*).

Table 1.1: Common molecular genetic abnormalities, tumor grade and 5 year survival rate.

Glioma subtype	Genetic abnormalities[a]	WHO tumor grade	5-year survival rate, %[b]
Oligodendroglioma	Chromosome 1p loss Chromosome 19q loss	II	62
Anaplastic oligodendroglioma	Chromosome 1p loss and/or chromosome 19q loss plus: Chromosome 9p loss *CDKN2A* mutations Chromosome 10 loss Chromosome 7 amplification	III	38

Increases in nuclear pleomorphism and hyperchromatism, as well as pronounced hypercellularity, brisk mitotic activity, prominent microvascular proliferation, and/or spontaneous necrosis, results in a picture that is histologically classified as anaplastic oligodendroglioma (malignancy grade III). Anaplastic forms of oligoastrocytomas (*Figure 1.2*) also occur and similar criteria are used to distinguish them from oligoastrocytomas. Oligodendrogliomas show relatively specific genetic abnormalities that differ from the other gliomas. The losses on 1p and 19q are most common among the grade II oligodendrogliomas and are present in over 50% of anaplastic oligodendrogliomas (malignancy grade III). Oligodendrogliomas grade II also show methylation of p14ARF, over-expression of EGFR and both ligands and receptors of the platelet derived growth factor (PDGF) system.

Tumorigenesis is a multistep process that involves a series of genetic and epigenetic alterations, yet the current models of tumorigenesis and many cancer therapies are mainly based on the results of the genetic approach. Epigenetic modification, such as aberrant methylation of normally unmethylated cytosines within CpG islands, is also a prevalent gene inactivation mechanism in human sporadic cancer [11]. Typically, CpG island methylation is assessed on genes known to play roles in tumorigenesis, especially in tumor samples where those genes do not have genetic alterations. This approach has identified many genes that are silenced by aberrant methylation including genes involved in differentiation, cell growth, apoptosis, and tumor progression.

MATERIALS AND METHODS

ANTIGEN PREPARATION USING HUMAN BRAIN TISSUE:

Brain Tissue after Antigen processing is mixed thoroughly with Freund's Complete Adjuvant [40% v/v]. Both are mixed thoroughly for 15 minutes in sterile injection vial. Adjuvant mixture is filtered through bacterial filter. The filtered mixture can now be termed as Antigen which can be taken for immunization procedure. This antigen preparation is distributed into sterile injection vials as per the requirement and dispersed to animal house.

IMMUNIZATION PROCEDURE OF RABBIT:

New Zealand White rabbit was used herein as subject and then complete Freund's adjuvant (CFA) initially, was then followed by Incomplete Freund's Adjuvant (IFA) for all subsequent injections. The Immunogen was diluted to 0.5 ml with sterile saline and combined with 0.5 ml of the appropriate adjuvant. The Immunogen and adjuvant were mixed thoroughly to form a stable emulsion that was injected beneath the skin of the rabbit (subcutaneously) in the area around the shoulders and intra-muscularly into the large muscle of the rear legs. About 1/4th of the antigen was used in each area. The method enhances the immune response from the sustained presentation of the Immunogen. Now the blood was collected from the central ear artery with a 19-gauge needle and allowed to clot and retract at 37oC overnight. The clotted blood was then refrigerated for 24 hours before the serum was decanted and clarified by centrifugation at 2,500 rpm for 20 minutes (*Figure 1.3*).

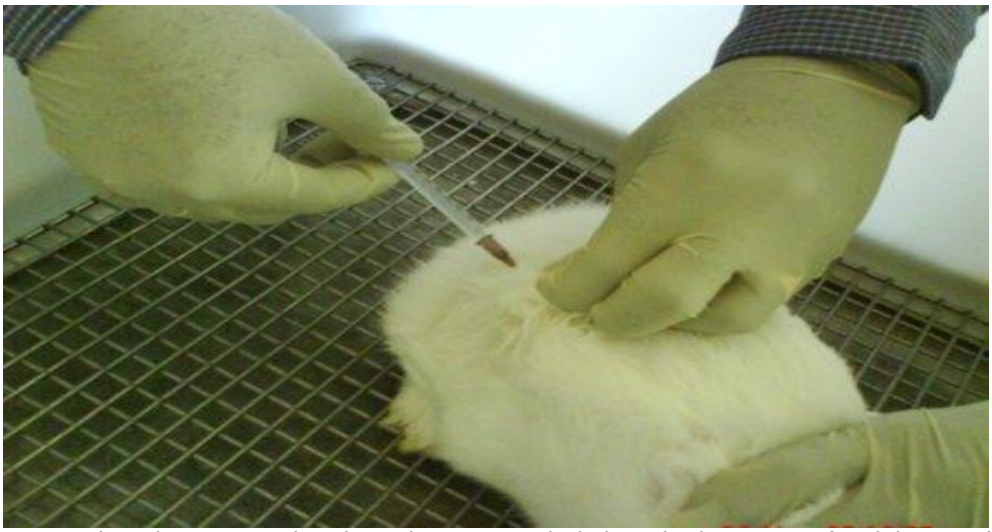

Figure 1- Inject the prepared antigen, intra-muscularly into the large muscle of the rear legs

After the immunization protocol, we are supposed to collect the bleeding of the rabbit and from that we are going to collect the plasma. Extraction of rabbit antibodies from collected plasma is been carried out by using the protocol given below. SDS-PAGE is carried out.Cross reactivity of antibodies with antigen is done by double immunodiffusion (Ouchterlony) followed by ELISA. These are the various assays carried out for estimation of production of polyclonal antibodies against cerebral tumor.

BLOOD COLLECTION AND PLASMA SEPARATION:

Collecting blood by lacerating ear or tail vessels is prohibited. There is always the potential that an artery will be lacerated rather than a vein, resulting in severe hemorrhage. In addition, these procedures are more painful than puncture with a needle because of the prolonged time for wound healing. Also, the site of the procedure is very susceptible to infection, hemorrhage and other complications. Regardless of the method of collection used, an animal may not be returned to its cage until complete hemostasis has been achieved (there is no more blood coming from the collection site). Hemostasis should be achieved using gauze and direct pressure. Up to several minutes of pressure may be required following arterial puncture. As a single blood draw, a maximum of 1% of the animal's body weight can be removed. If blood must be drawn more frequently than once every two weeks, a total of 0.5% of the animal's body weight can be removed each week with this total volume being spread out over the entire week if needed.

Animal Blood is carefully collected carefully in the presence of an anticoagulant [Tri sodium citrate] and it is kept undisturbed in refrigerator for a whole night. The next day a light yellowish brown colored supernatant is observed over the RBC mass in collection vial. If any cloudy substance is observed in the vial especially in the supernatant part then supernatant should be drawn into a centrifuge tubes and centrifuged at 3000-4000 rpm for 5 minutes. Now this clear supernatant is plasma which can be used for the further procedures.

IMMUNOGLOBULIN EXTRACTION USING DOUBLE PRECIPITATION REACTION:

To 0.5ml Plasma , 0.5ml Phosphate buffer was added into a test tube and freezed for 10min [0 - 4 degrees centigrade] . To the above mixture, pre-cooled Ammonium sulphate [0.5g] was added and freezed for 10min [0 - 4 degrees centigrade]. Now, this was mixed thoroughly for 10 minutes and then centrifuged at > 10000rpm for 5min. Pellet was collected and supernatant was discarded. Now, to the pellet, 12% Octanoic acid [Caprylic acid] was added and freezed for 10min [0 - 4 degrees centigrade]. Again, it is mixed thoroughly for 10 minutes, supernatant was collected and from this lower pellet and upper pellet were saved for further analysis. For this sample treatment we had used [1:2 ratio of the above supernatant & treatment buffer (SDS PAGE)]. This was followed by heating for 5min in boiling water bath and then the samples were loaded for SDS-PAGE.

ULTRAPURIFICATION OF PRECIPITATED IMMUNOGLOBULIN USING DIALYSIS:

The precipitated sample was taken into the bag and it was tied tight on the both the ends with sterile thread so that nothing can not ooze out. Here in this study the dialysis bag used has a cut off value

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between 12000 to 14000 Daltons. Hence the main agenda behind performing this is to get rid of unwanted proteins and excess of PEG which now in this step called as a contaminant. A minimum amount of the precipitated sample was taken into the dialysis bag and then it was kept in a beaker containing Phosphate buffer. This mixture was now mixed for whole night. On the next day the buffer present in the beaker was drained and then fresh buffer was filled in it. Now this was mixed for another whole night. Such Buffer changes should be given at least 3 to 5 times for a better dialysis process. A visual change can experienced by observing the sample present in the dialysis bag before the buffer washes and after the washes.

EVALUATION TECHNIQUES:

SDS- PAGE OF ULTRA PURIFIED IMMUNOGLOBULIN: This is a general protocol that was developed for the ISCO electroeluter but could easily be applied to other systems [12] . The procedure is then followed by Coomassive blue staining for the visibility of the bands.

DOUBLE IMMUNODIFFUSION [Ouchterlony]:

Precipitation occurs with most antigens because the antigen is multivalent (i.e. has several antigenic determinants per molecule to which antibodies can bind). Experimentally, an increasing amount of antigen is added to a constant amount of antibody in solution, initially at low antigen concentration, all of the antigen is contained in the precipitate. This is called the antibody-excess zone (i.e. prozone phenomenon). As more antigen is added, the amount protein precipitated increases until the antigen/antibody molecules are at an optimal ratio. This is known as the zone of equivalence or equivalence point. When the amount of antigen in solution exceeds the amount of antibody, the amount of precipitation will decrease. This is known as the antigen excess zone [13].

ELISA:

Enzyme-linked immunosorbent assay (ELISA), also known as an enzyme immunoassay (EIA), is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. The ELISA has been used as a diagnostic tool in medicine and plant pathology, as well as a quality control check in various industries. In simple terms, in ELISA, an unknown amount of antigen is affixed to a surface, and then a specific antibody is applied over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal [14].

RESULTS:

Evaluation for SDS PAGE: At the completion of electrophoresis followed by staining, a prominent 50kDa band was observed in the sample lane corresponding to the molecular weight marker lane , hence depicting a high titre of Immunoglobulin G in the sample . Also a light 85-90KDa band was observed thus showing the presence of a low titer of Immunoglobulin M. (*Figure 1.4*)

Evaluation for Double Immunodiffusion : The formation of the precipitin band between the antigen and the corresponding antibody is largely dependent on the concentrations of the reactants. If the system is balanced the precipitin band will be formed between the two wells. If the precipitin band is close to the antigen well and far from the antibody well then we can interpret that the concentration of the antibody is more and the antibody needs to be diluted. Diluent to be used here is7% Bovine Serum Albumin. If the precipitin band to the antibody well and far froe the antigen well then we can interpret that the concentration of the antigen is more and less antigen needs to be put or the antibody loaded should be more concentrated or undiluted. Since Snake venoms are Polyantigens many precipitin bands can be seen,this signifies that the animal has developed antibodies aginst many of the components of the venom. It should be noted that a distance of 5mm be kept between the antibody and the antigen well for proper diffusion. After the antigen and the antibody has been loaded the plate should be kept in a moist chamber but not in the fridge. The precipitin bands will appear with in 6hrs after loading the slide. If the precipitin rings do not appear after 12hrs,then the slide is kept in 1%Tannic acid solution (i.e.1gm Tannic acid in 100ml D/W) until the precipitin rings develop. Do not keep the slide for a long time in Tannic acid or the whole slide will become opaque. If the slide is put in Tannic acid it cannot be stained using Coomassie Blue stain.(*Figure 1.5*)

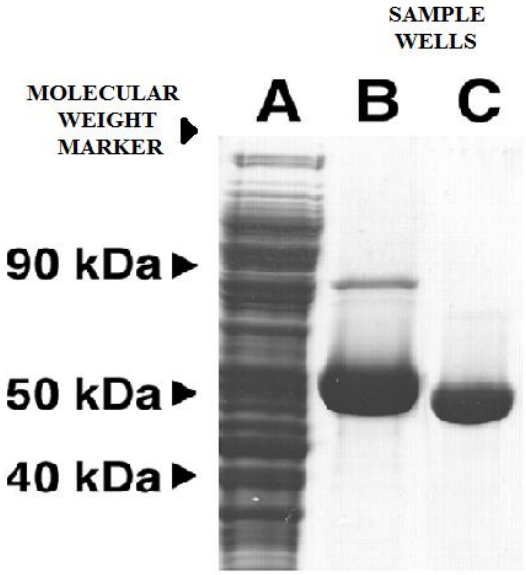

Figure 2- The bands corresponding to their molecular weight markers are seen, with the band corresponding to 50kDa was prominent in the sample lanes.

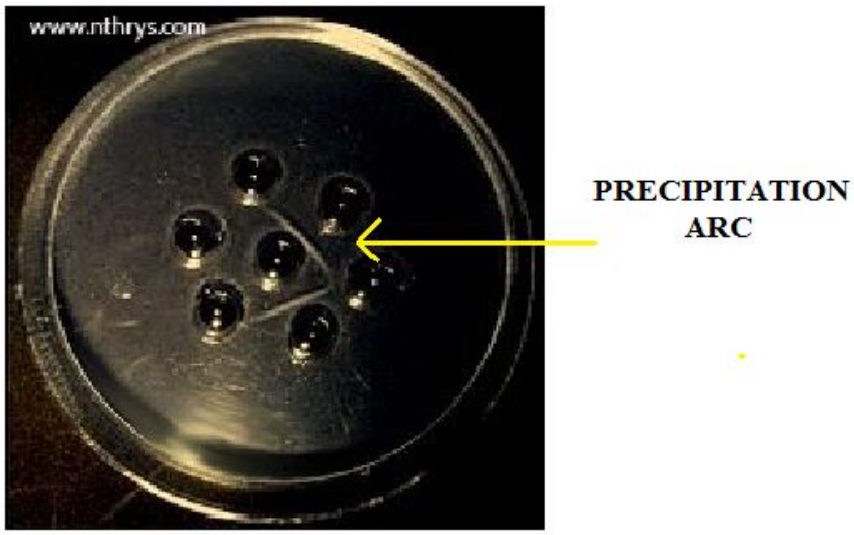

Figure 3- Center well is loaded with antigen and the surrounding wells are loaded with antibodies isolated from various batches from animal bleed.

Evaluation for ELISA: We consider the described ELISA for the quantification of polyclonal antibodies as a useful tool for the determination of polyclonal antibodies in amniotic fluid in dialyzed sample. Its most promising application is expected in the diagnosis of cerebral cancer and therby its effective treatment. The antibodies generated are applicable to multiple techniques.(*Figure 1.6)*

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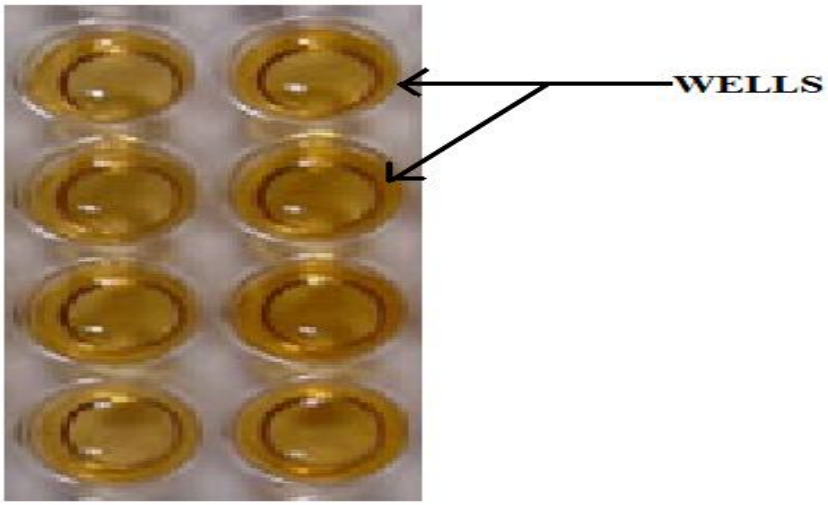

Figure 4- Wells showing a visible coloured reaction, indicating that there was formation of antigen-antibody complex.

DISCUSSION

In this project entire concentration is laid on qualitative approach. Hence antigen preparation and immunization protocols are studied in detail. Oligodendrogliomas antigen samples selected in this project is mass of brain tissue excised from respective patients during surgical removal of tumour.This source is been collected for this study by the help of biohazard department in collaboration with hospitals.Antigen processing is performed in the above said process. Successive immunization schedule is followed. Rabbit is used as a host in this project. After successive immunization schedule plasma is procured from rabbit blood and IgG extraction procedure is performed and purification is further followed by dialysis protocol. The dialysis bag's porosity selected for this study is ±12,000 daltons. Dialysis's process is bit prolonged to 3-4 days for a better purification of IgG from smaller proteins. As the precipitation agents' percentage in precipitation reaction enables us to get rid of higher molecular weight proteins(<10,00,00 daltons). Dialysis will help us to get rid of lower molecular weight proteins (12,000 daltons). As IgG(antibody) is having 1,50,000 daltons and they are very less number of proteins around this molecular weight(nearly negligible), purification of IgG with this two step process can be achieved.

CONCLUSION

By the above results, we can conclude that antibody is being produced against oligodendrogliomas cell suspension (antigen) successfully developed. Further quantitative analysis is required to study ethicacy and evidity of antibody.

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