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Response to "Human cytomegalovirus infection in tumor cells of the nervous system is not detectable with standardized pathologico-virological diagnostics"

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See the article by Baumgarten et al, on pages 1469–1477.

This manuscript by Baumgarten et al again challenges the existence of human cytomegalovirus (HCMV) infection in glioblastoma (GBM). The findings were first reported by our group in 2002.[1] Before then, there had only been one case report of immunohistochemical (IHC) confirmation of HCMV infection in GBM, and that was in an AIDS patient.[2] Since the 1980's, several groups have shown that CMV could persistently infect fetal human brain, immature glial cells, and GBM cells in culture.[3–6]

After our first report, several groups were unable to confirm our IHC findings.[7,8] We believe this was due to the fact that our extensively optimized and sensitive protocols deviated significantly from simpler techniques used for routine CMV immunodetection in AIDS and transplant patient tissues. As a result, when investigators adopted our techniques, their results have been consistent with ours;[9–11] when they have not used our techniques, their results have usually been negative. Historically, it is not uncommon for new, complex techniques to be controversial and vary widely. This was the case with Her-2 immunostaining in breast cancer in the early years, which was quite controversial and was only accepted when performed on frozen sections until a widely consistent protocol could be developed for formalin-fixed paraffin-embedded (FFPE) sections.[12]

Our sensitive CMV protocols have been published, and we are in the process of developing an automated IHC and in situ hybridization (ISH) reference lab to perform these studies.[13] Critical steps that are involved in these IHC protocols for FFPE GBM specimens include the following:

(1) IHC on sections older than 6 months requires postfixation in formalin; otherwise, results are likely to be negative.
(2) After rehydrating in graded alcohols, it is critical that the slides be digested in trypsin or pepsin. If this step is omitted, the results will always be false negative.
(3) Antigen retrieval in citrate buffer at 90°C for 4 minutes followed by 45°C for 2.5 hours is essential for breaking the formalin-induced protein cross-links, which will unmask the CMV antigens and epitopes. Elimination of this step will result in false-negative results.
(4) Blocking of endogenous peroxidases with H2O2 3% for 12 minutes at room temperature is required to prevent background staining.
(5) To prevent nonspecific antibody binding, we employ an Fc receptor blocking step prior to the addition of primary antibody.
(6) We apply primary antibody at 4°C overnight. If primary antibody is applied for only 30 minutes at room temperature, we will get nonspecific background and false-negative results.

Based on the methods described in the manuscript by Baumgarten et al. none of the above steps, which we have found to be critical, were employed. In our experience, elimination of these steps will almost certainly result in negative results, consistent with those reported here. Regardless of the antibody concentration, if the epitopes are not unmasked by citrate and protein digestion, they will not be detected.

Other issues addressed in this manuscript include:

Positive Control

The positive controls used in this manuscript do not address the essential question of low copy protein level. Cells infected in tissue culture with CMV will be prone to high-level viral protein expression, and thus it is easy to identify one positive cell in a million. This is not relevant to the question being asked, which is "does the test identify endogenous low copy CMV infection in brain?" The CMV-infected cerebellar tissue is a reasonable positive control, but it is likely that even more immunoreactivity could be apparent if our published protocols were used, as we demonstrated in our published methods paper about performing IHC on known infected lung with routine and sensitive techniques.[13]

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Polymerase Chain Reaction Results

We agree with the authors that PCR for CMV DNA from paraffin sections is often difficult. Our own group has had significant difficulty with this aspect of detection. Nevertheless, we have been successful in performing laser capture on CMV-positive areas of GBM cases (based on IHC) as well as amplifying and sequencing viral genome from tumor cells. Ranganathan et al have addressed these issues,[14] and they found that almost all GBM specimens were positive for CMV DNA, PCR detection was highly correlated with age of specimens, and frozen sections were much more likely to allow CMV detection than older FFPE specimens. Bhattacharjee et al confirmed these findings and also demonstrated evidence of multiple CMV proteins in GBM tumors by Western blot.[15]

Serological Data

The authors found that the CMV serological status of patients is not associated with survival. For a decade, various experts have debated the importance of CMV serological status in GBM patients. Importantly, Bianchi et al recently confirmed a high prevalence of CMV in GBM based on IHC and PCR using techniques similar to those we published, but they also found that a significant percentage of these same patients were CMV seronegative.[16]

Moving Forward

Reconfirming negative results using techniques that are known to be below the level of sensitivity required for detecting CMV in FFPE GBM specimens, based on several publications only, delays progress in the field. Our group has had the most success in performing IHC and ISH on frozen sections of GBM specimens or primary GBM cultures when at passage 0–2. We and others have routinely performed Western blots on GBM frozen specimens and primary cultures and confirmed the existence of CMV proteins in these tumors.[9,15,17–19] Indeed, we have been able to knock down individual CMV proteins in primary human GBM cultures using siRNAs targeting specific CMV genes [19] (and manuscript in preparation). Finally, CMV-based immunotherapy protocols in GBM have indirectly confirmed CMV antigens in GBM specimens[20,21] and thus demonstrate great promise as novel therapies for GBM. It is therefore the opinion of this investigator that future studies to detect CMV in GBM should be based on confirming techniques that have been proven to work instead of reattempting to confirm negative results using techniques that have proven not to work.

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