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# Evaluation of polyomavirus BK cellular immune response by an ELISpot assay and relation to viral replication in kidney transplant recipients

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**SUMMARY**

The failure of immune surveillance may be associated with polyomavirus BK reactivation, potentially leading to the development of nephropathy in kidney transplantation. BK-specific cellular immune response may be used to modulate immunosuppressive therapy, but few studies have investigated the topic. Herein, we serially evaluated BK-specific response in 149 kidney transplant recipients and found that only 14/149 (9.4%) were responders. Episodes of viral reactivation (viremia and/or viruria) occurred only in non-responder patients. The frequency of BK-specific immune response appears to be lower than that for other persistently infecting viruses such as cytomegalovirus.

*KEY WORDS:* Polyomavirus BK, Elispot assay, Kidney transplantation.

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## SHORT COMMUNICATION

Polyomavirus BK-associated nephropathy (PVAN) is a major cause of poor renal graft survival. PVAN has been reported in from 1% up to 10% of kidney transplant recipients (Hirsch *et al.,* 2005; Hirsch *et al.,* 2006; Giraldi *et al.,* 2007), particularly in the first year post-transplantation. Therapeutic options are limited due to the unavailability of specific anti-viral drugs. Therefore, a pre-emptive strategy consisting in the monitoring of viral replication and modulation of immunosuppressive protocols in the presence of BK reactivation is the only available method for clinical management of high-risk patients (Costa *et al.,* 2008). The loss of the transplanted organ with a return to hemodialysis has been reported in 30%-80% of PVAN cases (Cavallo *et al.,* 2009).

A number of studies have shown that the control of viral replication and of the onset of PVAN is related to the development and/or reconstitution of BKV-specific cellular immune response, whereas the lack of protective immunity may favour the onset of active infection and progression to PVAN (Comoli *et al.,* 2004; Chen *et al.,* 2006; Binggeli *et al.,* 2007; Prosser *et al.,* 2008; Schachtner *et al.,* 2011). As the failure of immune surveillance has been shown to play a role in the onset of viral replication, monitoring strategies also involving the evaluation of virus-specific immune response should be recommended, as reported for other persistently infecting viruses like cytomegalovirus that can be associated with major morbidity and mortality in transplant recipients. Nevertheless, very few centres perform routine immunological monitoring of polyomavirus BK and data on the status of BKV-specific cellular immune response are limited.

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This study investigated BKV immunological status by an ELISpot assay and evaluated the prevalence of BK viremia and viruria by real-time PCR in a population of kidney transplant patients routinely evaluated in the first year post-transplantation.

The study population consisted of 149 adult kidney transplant recipients (M/F, 80/69; mean age ± standard deviation, 53.2±14.3 years; range, 28-77) evaluated at the Renal Transplant Unit and the Post-Renal Transplant Outpatient Unit of the Azienda Ospedaliera Città della Salute e della Scienza of Turin over a period of 18 months. Immunosuppressive protocols were as follows: tacrolimus (Tac), mycophenolate mofetil (MMF) and steroid in 78 patients; Tac and Steroid in 26; cyclosporine A (CyA), MMF and steroid in 17; CyA and steroid in eight, and other protocols in 20. According to our centre's practice, virological monitoring was performed by evaluation of BK viral load on serum and urine specimens every two weeks up to month 3 post-transplantation, then every three months; whereas viro-immunogical monitoring was performed monthly up to month 3 post-transplantation, then every three months (Cavallo *et al.,* 2009).

Viremia and viruria were evaluated by a commercially available real-time TaqMan PCR assay (BKV ELITe MGB™ kit, Elitech Group, Milano, Italy) using the 7500 real-time PCR system instrument (Applied Biosystems, Life Technologies Italia, Monza, Italy), following semi-automated extraction with the EasyMag instrument (Biomerieux, Marcy l'Etoile, France), according to the manufacturer's instructions.

The ELISpot assay was performed as previously described (Costa *et al.,* 2012). Briefly, automated separation of total CD3+ cells from whole blood specimens was performed with the RoboSep® cell separator (StemCell Technologies, Vancouver, Canada) using the EasySep negative selection protocol, following the manufacturer's instructions. An aliquot of 200,000 cells was used for the ELISpot assay (ELISpot Interferon-γ Basis kit; AID, Strassberg, Germany) and incubated on anti-IFN-γ coated wells of a microplate with a BKV-specific peptide mix including nine peptide sequences from VP1 and large T-antigen, as previously reported (Krymskaya *et al.,* 2005; Chen *et al.,* 2006; Li *et al.,* 2006; Randhawa *et al.,* 2006) (supplied by Elitech Group). Following 18-24 hours incubation at 37°C in a $CO_2$ incubator, antigen-induced production of IFN-γ was visualized by an enzyme-labeled detection antibody, with each coloured spot representing a single cell secreting IFN-γ. For negative and positive controls, cells were incubated with RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) alone and with phytohemagglutinin mitogen, respectively. Results were analyzed using a computer-assisted system (AID ELISpot Reader System, AID). Specific immune response was expressed as absent or weak (non-responder) in the presence of a number of spot forming units <5/200,000 cells and present (responder) for >5/200,000 cells.

Data are expressed as raw numbers, percentages, and means, as appropriate. For statistical analysis, the chi square and the Fisher's exact tests were used, as appropriate. A p value <0.05 was considered statistically significant.

Overall, 975 serum and 975 urine specimens were evaluated during the study period, whereas 305 whole blood specimens were collected for ELISpot assay. A complete series of specimens for ELISpot assay was not available in all the patients due to missing sending or inadequacy of sample (invalid negative or positive control, insufficient number of cells). Viremia and viruria were found in at least one determination in 12 (8.0%) and 32 (21.4%) of 149 patients, respectively. Viral load was $<10^4$ copies/ml serum in all the patients, except two; viruria was $>5x10^6$ copies/ml urine in six cases. No patient was diagnosed with a PVAN, although one patient presented viremia and viruria values higher than $1.6x10^4$ copies/ml and $5x10^6$ copies/ml, respectively. These values were persistent throughout the study period, although in the absence of an increase in serum creatinine.

Considering viro-immunological monitoring (Table 1), only 14 (9.4%) patients were responders (at least 5 spot forming units/200,000 cells; mean, 7, range 5-23). None of the responder patients showed viral reactivation (viremia and viruria were always negative at follow-up; p = not significant). The patient with persistent high level viral replication was persistently a non-responder at three evaluations (number of spot forming units/200,000 cells =0).

TABLE 1 - *Results of virological and viro-immunological evaluation of polyomavirus BK in sthe tudy population of kidney transplant recipients.*

| *Total Patients N = 149* | *Polyomavirus BK-DNA* | | |
|---|---|---|---|
| | *Negative* | *Viremia* | *Viruria* |
| BK-virus cellular immune response status | | | |
| Responder N = 14 | 14 (100%) | 0 | 0 |
| Non-responder N = 135 | 103 (76.3%) | 12 (8.9%) | 32 (23.7%) |

Two main conclusions can be drawn based on the results of this study. First, the prevalence of a responder status specific for polyomavirus BK in renal transplant recipients evaluated in the first year post-transplantation is relatively low. In fact, a responder status was found in only 9.4% of the patients; this value is much lower than that found for other viruses potentially implicated in transplant morbidity, such as cytomegalovirus. This could be explained by a less frequent exposure to the viral agent, as hypothesizable for herpesviruses in comparison to polyomaviruses. For cytomegalovirus, it has been demonstrated that viral exposure can boost the immunogical response (Abate *et al.,* 2010). This was not found in our study, in which none of the responder patients exhibited an episode of viral replication during the study period. Another interesting observation for BKV-specific response in comparison to cytomegalovirus consists in the fact that the overall frequency of virus-specific cells was much lower (Costa *et al.,* 2012). This could be due to the use of a weak stimulus: it could be hypothesized that the peptides used for antigen stimulation are not the most relevant and immunogenic. This could be overcome by performing studies specifically addressed to the evaluation of extensive peptide libraries or viral lysates. Previous studies have investigated BKV-specific cellular immune response in different types of individuals. Zhou and colleagues (Zhou *et al.,* 2007) found that healthy BK-seropositive subjects possess populations of both CD4+ and CD8+ T cells specific for Bk antigens (including large T antigen and VP1) and observed that CD4+ T cells may play a role in maintaining memory responses to BK and contribute to immune control of viral replication. Other authors found that control of BK replication and the onset of PVAN is correlated with the development or reconstitution of specific cellular immunity, whereas the lack of protective immunity may favour the occurrence of active infection and progression to PVAN (Comoli *et al.,* 2004; Binggeli *et al.,* 2007). In one study (Comoli *et al.,* 2004), while the mean number of spot forming units (interferon-gamma secreting cells)/$10^6$ peripheral blood mononuclear cells was 151 in BK-seropositive healthy subjects versus 5.5 in pediatric kidney transplant recipients with viruria and good renal function, no spot forming unit was detected in renal transplant patients with PVAN. An increase in the number of interferon-gamma secreting cells to levels similar to healthy subjects occurred in PVAN after immunosuppression reduction in concomitance with a reduction of viremia and viruria. In another study (Binggeli *et al.,* 2007), cellular response was significantly lower (to both large T antigen and VP1) in kidney transplant patients with increasing or persistent viral load in comparison to those with decreasing load or past PVAN.

A limitation of the present study is represented by the low number of patients with viral reactivation at risk of nephropathy: in particular, only one patient presented viral loads in urine and plasma consistent with those found in the presence of a suspected nephropathy, although persistently in the absence of impaired renal function. It should be noted that the Renal Transplant Centre of Turin, as previously reported (Costa *et al.,* 2008), has a very low incidence of PVAN, probably due to the close monitoring and subsequent immunosuppressive therapy modulation in the presence of viral reactivation. It is likely that a prompt modulation of immunosuppression, following the detection of viremia values <$10^4$ copies/ml serum as in our population, results in some recovery of BKV-specific immune response. In addition,

viral exposure could represent a potential immunological boost in this context, therefore preventing the subsequent development of virus-related renal damage. In this connection, further studies should be performed on the impact of different immunosuppressive protocols, as suggested by previous studies in which BK-specific responses were inversely correlated with trough levels of tacrolimus, but not other drugs (Egli *et al.,* 2009).

Taking these limitations into account, this study suggests that the occurrence of a responder status is associated with control of viral replication, whereas reactivation may occur in the absence of BKV-specific cellular immune response. The limited number of responder patients limited the statistical significance of these data, however it is likely that larger studies will provide more relevant data.

No definitive approach has currently been defined on the basis of the evaluation of BKV-specific cellular immune response at our centre. One important issue could be the prompt initiation of modulation of immunosuppressive protocols in non responder patients and, on the other hand, the re-evaluation of BK viremia within a few days/weeks in responder patients before a pre-emptive reduction of immunosuppression.

Further studies on this approach to clinical management, as well as the impact of the type of immunosuppression, investigating some technical issues could lead to an improvement in the clinical usefulness of the immunological monitoring of polyomavirus BK in renal transplant recipients.

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