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SHORT COMMUNICATION

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Examination of control asymptomatic cohorts reveals heightened anti-EBV and HHV-6 A/B dUTPase antibodies in the aging populations

Khaled Alharshawi[1] | Brandon Cox[1] | Maria E. Ariza[1,2]

[1]Department of Cancer Biology and Genetics, The Ohio State University Wexner Medical Center, Columbus, Ohio, USA

[2]Institute for Behavioral Medicine Research, The Ohio State University Wexner Medical Center, Columbus, Ohio, USA

Correspondence
Maria E. Ariza, Department of Cancer Biology and Genetics, The Ohio State University Wexner Medical Center, Columbus, OH 43210, USA.
Email: maria.ariza@osumc.edu

Present address
Khaled Alharshawi, Division of Digestive Diseases and Nutrition, Section of Hepatology, Rush University, Chicago, IL, USA.

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Abstract

Members of the human *Herpesviridae* are found in high prevalence in the human virome. While these viruses are known to cause numerous disease pathologies in symptomatic individuals little is known concerning the role that these viruses may have in modulating the host immune system in asymptomatic "healthy" individuals, especially during the aging process. Examination of three cohorts of "healthy asymptomatic" individuals ($n = 255$) for the presence of antibodies against the herpesviruses deoxyuridine triphosphate nucleotidohydrolase (dUTPase) as a marker for lytic/abortive-lytic replication demonstrated that all cohorts exhibited differential anti-herpesvirus dUTPase antibodies positivity frequencies ranging from 40.4% to 84% with some individuals in these cohorts expressing antibodies to the dUTPases of multiple herpesviruses (17.2%–56%). Furthermore, our results demonstrate that there was a statistically significant difference in anti-human herpesvirus 6 A and 6B (HHV-6 A/B) dUTPase antibodies in Cohort 3 (age = 66.2 ± 15.02 years) versus Cohort 1 (age 46.88 ± 8.61 years), suggesting that reactivation of HHV-6 A/B is not attenuated by aging. It is well established/documented that herpesvirus dUTPases induce immune dysfunction, as such it is of critical importance that additional studies be performed to determine how these viral proteins alter immune responses in asymptomatic individuals.

KEYWORDS
aging, anti-deoxyuridine triphosphate nucleotidohydrolase (dUTPase) antibodies, asymptomatic "healthy" individuals, Herpesviruses, immune dysfunction, reactivation

1 | INTRODUCTION

The virome of humans is complex and is composed of numerous virus families including members of the Herpesviridae. The Herpesviridae are classified into three subfamilies: the α-herpesviruses (herpes simplex types 1 and 2 [HSV-1 & 2] and varicella-zoster virus [VZV]), the β-herpesviruses (human cytomegalovirus [HCMV], human herpesvirus 6 A and 6B [HHV-6A & 6B] and human herpesvirus-7 [HHV-7]) and the γ-herpesviruses (Epstein-Barr virus [EBV] and human herpesvirus-8 [HHV-8]). While these viruses can be distinguished based upon cellular tropism, serology, and DNA sequence, a common feature shared by the members of the *Herpesviridae* family is that they establish life-long

persistent infections in individuals. These viruses are highly prevalent in the human population and are associated with numerous diseases in immune-competent and immune-suppressed individuals.

However, studies are severely lacking in examining/addressing what role(s) herpesviruses may have in asymptomatic "normal" individuals and how proteins encoded by these viruses modulate the host immune system? While most studies focus on the "bipartite" life cycles of these viruses, which alternate between latent and lytic phases, several studies have demonstrated that abortive-lytic replication is common with herpesviruses.[1,2] Most notably abortive-lytic replication of EBV has been implicated in the pathophysiology of infectious mononucleosis,[1] chronic active EBV infections,[3] and EBV-associated malignancies.[3,4] Also, it would be important to study in asymptomatic "normal" individuals what is the effect(s) of coinfections not only with other herpesviruses but also with other members of the host own virome[5–7]? What effect does aging have on the expression of specific virus proteins and the host response to these viruses? Studies have suggested that immune senescence which occurs during aging may result in increased replication of viruses. This premise is supported by studies with EBV and HCMV demonstrating increased antibody formation against lytic proteins in older populations[8,9] as well as alteration in B and T-cell populations, suggesting a lack of immune control.[10–12] Interestingly, a recent study by Kobayashi et al. reported that HHV-6B reactivation is attenuated by aging in a cohort of asymptomatic individuals.[13] A limitation of this study however is that serological studies were performed using an enzyme-linked immunosorbent assay (ELISA) for the detection of immunoglobulin G (IgG) against an unreported HHV-6 antigen. The study also found that the amount of HHV-6 DNA in saliva negatively correlated with age. However, it is important to keep in mind that *viral load is not a true indication of reactivation since abortive-lytic replication does not result in increased viral load*. Altogether these results suggest that the aging process may have different effects on the various herpesviruses.

To address this possibility, we examined the sera from three separate cohorts of "healthy controls" for antibodies to the herpesviruses' deoxyuridine triphosphate nucleotidohydrolase (dUTPase) protein, a marker of lytic/abortive replication, expressed as an early protein in EBV (BLLF3), HSV-1 (UL50), VZV (ORF8), and HHV-6A/B (U45). Our study demonstrates that approximately 18% of the individuals in two cohorts (1 and 3), that differed primarily in age, were simultaneously expressing anti-dUTPase antibodies to several herpesviruses. Furthermore, the percentage of patients exhibiting anti-HHV-6A/B antibodies in the older cohort (Cohort 3) was higher than that of patients in the youngest cohort (Cohort 1) suggesting that the reactivation of HHV-6A/B is not attenuated during aging.

2 | MATERIALS AND METHODS

2.1 | Study subjects, patient consent, and ethical review

All human serum samples ($n = 255$) used in this study were deidentified and the acquisition of the deidentified sera was approved by the Institutional Review Board at The Ohio State University. Human blood sample collection and all experimental procedures were approved by the Institutional Review Boards at the respective institutions, which include Nova Southeastern University, Institute for Neuro Immune Medicine Fort Lauderdale, Florida and Center for Infection and Immunity, Columbia University, New York, and the European sample collectors in the EPYLYMPH study/the NIH cancer repository control samples. Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. Further detailed information regarding patient demographics and inclusion/exclusion criteria for the recruitment of subjects has been described elsewhere.[6,14,15] The cohorts used in this study were designated as "healthy controls" in the previous studies.

2.2 | dUTPase ELISA assays

Herpesviruses dUTPase ELISAs were performed as described previously.[4] Briefly, Nunc-Immuno Plate MaxiSorp 96-well plates were coated overnight at 4°C with recombinant dUTPase protein at 2.5 µg/ml in phosphate-buffered saline (PBS). Plates were washed on a Biotek ELx50 plate washer three times with PBS/0.05% Tween 20 and blocked with blocking buffer (PBS/2.5% bovine serum albumin [BSA]) at room temperature (RT). All serum samples were used at a 1:800 dilution in blocking buffer and incubated for 2 h at RT in duplicate. Plates were washed three times with PBS/0.05% Tween 20 followed by incubation with anti-human-IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma Chemical Co.) at 1:1000 dilution in blocking buffer at RT for 1 h. Plates were washed six times with PBS/0.05% Tween 20 and incubated for 15 min with 100 µl of OPD substrate (Invitrogen). Sulfuric acid (50 µl of 2 M H_2SO_4) was added to stop the reaction and plates were read at 490 and 690 nm for background on a Lab Systems Multiskan MCC/340 plate reader using the Genesis v3.05 Life Sciences Ltd software. The background from the 490 nm uncoated wells and PBS-BSA (negative controls) was subtracted from the mean absorbance of the coated wells. A positive reaction was defined as a serum sample that led to a signal three times over the background OD of the control serum.

2.3 | Statistical analysis

GraphPad Prism 9 software (GraphPad Software) was used for all statistical analyses. To compare two groups a two-tailed Mann–Whitney *U* test was employed. A one-way analysis of variance (ANOVA) Kruskal–Wallis test was used for performing multigroup comparisons. Values of $p < 0.05$ were considered statistically significant.

3 | RESULTS

The data presented in Table 1 show the level of heterogeneity in the percentage of individuals positive for antibodies against the dUTPase protein of multiple herpesviruses observed among the three "healthy

TABLE 1 Anti-herpesviruses' dUTPase antibodies in three independent healthy, asymptomatic cohorts

dUTPase Ab[a] subgroup	Cohort 1 (n = 151)	Cohort 2 (n = 25)	Cohort 3 (n = 79)
Negative	88 (58.28%)	4 (16%)	38 (48.1%)
EBV only	15 (9.93%)	7 (28%)	0
HHV-6 only	20 (13.25%)	0	27 (34.18%)
HSV-1 only	ND	0	0
VZV only	2 (1.32%)	0	0
EBV + HHV-6	15 (9.93%)	14 (56%)	0
EBV + HSV-1	ND	0	0
EBV + VZV	1 (0.67%)	0	0
HHHV-6 + HSV-1	ND	0	4 (5.06%)
HHV-6 + VZV	2 (1.32%)	0	0
VZV + HSV-1	ND	0	0
EBV + HHV-6 + VZV	8 (5.30%)	0	0
EBV + HHV-6 + HSV-1	ND	0	7 (8.86%)
EBV + HHV-6 +HSV-1 + VZV	ND	0	3 (3.8%)
% Positive for 2 or more herpesviruses in total population	17.22	56	17.72
Age	46.88 ± 8.61	50.45 ± 9.37	66.2 ± 15.02
% Female	48	80	50

Abbreviations: EBV, Epstein-Barr virus; HHV-6, human herpesvirus 6; HSV-1, herpes simplex type 1; ND, not determined; VZV, varicella-zoster virus.

[a]Anti-herpesviruses dUTPase antibodies were determined by ELISA[4] as described in Section 2.

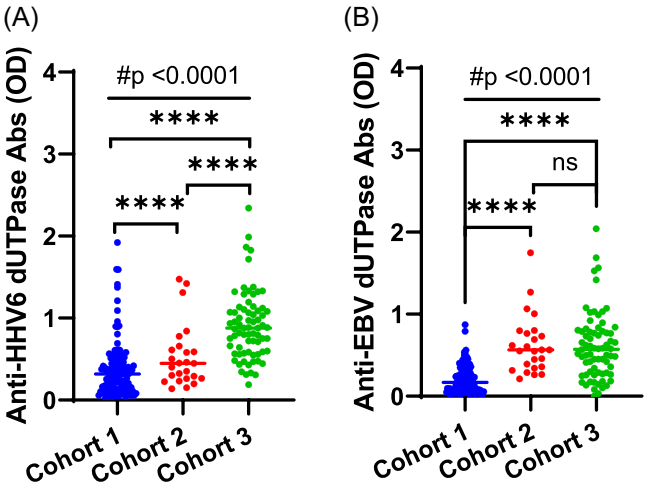

FIGURE 1 Anti-herpesviruses' dUTPase antibodies in asymptomatic healthy cohorts. Antibodies to the dUTPase of the human herpesviruses HHV-6 (A) and EBV (B) were determined using a recombinant dUTPase-specific ELISA[6] in three independent healthy cohorts (n = 151 Cohort 1, n = 25 Cohort 2, and n = 79 Cohort 3) with median age 46.88 ± 8.61, 50.45 ± 9.37, and 66.2 ± 15.02, respectively for Cohorts 1, 2, and 3. The data represent the mean ± SD of $n \geq 4$. To compare two groups a two-tailed Mann–Whitney U test was employed (****$p < 0.0001$, *ns = Not significant ($p = 0.757$)*. A one-way ANOVA Kruskal–Wallis test was used for a multigroup comparison (*#$p < 0.0001$)*. Values of $p < 0.05$ were considered statistically significant. ANOVA, analysis of variance; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; dUTPase, deoxyuridine triphosphate nucleotidohydrolase; HHV-6, human herpesvirus 6

control" cohorts examined (n = 255). Cohort 2 exhibited the highest percentage (84%) of subjects positive for anti-herpesviruses dUTPase antibodies compared with Cohort 1 (41.72%) and Cohort 3 (51.9%). Also, Cohort 2 had the highest percentage of subjects exhibiting antibodies against multiple herpesviruses dUTPases 56% versus 17.22% and 17.72%, respectively, for Cohorts 1 and 3. Since HHV-6 and EBV exhibit the highest prevalence in the human population, we examined the presence of antibodies against the dUTPases from these viruses alone and in combination with other herpesviruses. With respect to EBV, 84% of the subjects in Cohort 2 had anti-EBV dUTPase antibodies while subjects in Cohorts 1 and 3 had 25.83% and 12.66%, respectively. Conversely, 51.9% of the subjects in Cohort 3 exhibited anti-HHV-6A/B dUTPase antibodies while 29.80% and 56% were seropositive in Cohorts 1 and 2, respectively. As shown in Figure 1, there was a statistically significant difference between anti-HHV-6A/B dUTPase antibody levels in Cohort 1 versus Cohort 2, Cohort 1 versus Cohort 3, and Cohort 2 versus Cohort 3 ($p < 0.0001$ by two-tailed Mann–Whitney U test). Notably, while there was a statistically significant difference in seropositivity for anti-EBV dUTPase antibodies between Cohorts 1 and 2 and Cohorts 1 and 3 ($p < 0.0001$ by two-tailed Mann–Whitney U test), there was no statistical difference between Cohorts 2 and 3 ($p = 0.757$ by two-tailed Mann–Whitney U test). Multigroup comparisons also revealed a statistically significant difference in anti-HHV-6A/B and anti-EBV dUTPase antibody levels among the three cohorts ($p < 0.0001$ by one-way ANOVA Kruskal–Wallis test).

4 | DISCUSSION

While several studies have demonstrated the reactivation of multiple herpesviruses in patients with observable clinical features[16] and in subjects undergoing extreme stress, little is known concerning the expression of herpesvirus proteins in asymptomatic individuals or how these proteins may affect the immune status of the host. Because of the narrow host specificity of the herpesviruses, it is not possible to recapitulate the human herpesvirus virome in animal models and thus most approaches have employed either multiplex quantitative reverse- transcription polymerase chain reaction (qRT-PCR) or serology-based methods to examine the virome in humans. The major problem

with these approaches is that the genes/proteins targeted for detection in most cases are expressed late during lytic replication of the virus or in latency. This would not allow for the determination of abortive-lytic replication, and therefore may lead to the erroneous conclusion and/or assumption that these control cohorts are negative for virus reactivation. Recently, it was reported that there was a negative correlation between HHV-6 antibody titers and age in asymptomatic subjects using an HHV-6-specific IgG ELISA. However, the antigen employed in this system was not identified and since most adults have antibodies against HHV-6A/B proteins it is somewhat surprising that such a conclusion could be drawn.[16] This study also found that the amount of HHV-6 DNA in saliva negatively correlated with age. However, it is important to point out that viral load is not a true indication of reactivation since abortive-lytic replication does not result in increased viral load. Using antibodies against the early dUTPase protein (U45) our results demonstrate that the percentage of patients older than 50 (Cohort 3) is equal to or greater than those in the younger cohorts (Cohorts 1 and 2). Since the U45 gene encodes for an early protein this suggests that there is significant lytic/abortive-lytic replication occurring in this population. It is not possible to distinguish between HHV-6A and HHV-6B U45 dUTPases since these proteins exhibit greater than 94% identity/homology but if the results concerning HHV-6B are verified that would suggest that the increased positivity in dUTPase antibodies observed in our study in the older population must be due to the increased lytic/abortive-lytic replication of HHV-6A.

Our study also demonstrates that there is lytic/abortive-lytic replication of one or multiple herpesviruses, based upon the presence of anti-herpesvirus dUTPase antibodies, in the three cohorts of asymptomatic controls ranging from 41.72% in Cohort 1, which was the youngest population, to 84% in Cohort 2. The subjects in Cohort 2 appear to represent a unique cohort when compared with the other two used in this study. Cohort 2 had the highest percentage of subjects exhibiting anti-herpesvirus dUTPase antibodies and these antibodies were directed primarily against EBV. Also, this cohort had the highest percentage of females. It has been reported that in the case of EBV, females exhibit higher levels of EBV reactivation than males,[17,18] which may account for the increased levels of anti-EBV dUTPases antibodies in Cohort 2 when compared with Cohorts 1 and 3.

We have previously shown that herpesvirus dUTPases serve as pathogen-associated molecular pattern (PAMP) proteins with novel neuro-immunoregulatory functions.[19,20] The presence of antibodies against the EBV and HHV-6A/B dUTPases suggest that lytic/abortive-lytic replication is occurring in a subset of these asymptomatic "normal" individuals. This could result in a "heightened state" in their immune status, which will complicate the interpretation of studies concerning immune activation when using such cohorts. Thus, when conducting herpesvirus serology studies it would be important to use approaches that measure antibodies to both immediate early or early and late virus proteins.

AUTHOR CONTRIBUTIONS

Maria E. Ariza devised the study and obtained funding, analyzed data, and wrote the manuscript. Khaled Alharshawi and Brandon Cox conducted the experiments, analyzed data, and edited the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in PubMed ncbi at http://www.pubmed.ncbi.nlm.nih.gov

ORCID

Khaled Alharshawi https://orcid.org/0000-0002-2132-9695
Brandon Cox https://orcid.org/0000-0001-6753-6211
Maria E. Ariza http://orcid.org/0000-0003-2560-5367

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