Brief Report

Case Study of Herpesvirome: Individual Two Waves of the Immunoreconstitution and Acquisition of B Cell Memory May Be Observed in Various Specific IgG Kinetics after Hematopoietic Stem Cell Transplantation

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Posted Date: 22 December 2023

doi: 10.20944/preprints202312.1765.v1

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Case Study of Herpesvirome: Individual Two Waves of the Immunoreconstitution and Acquisition of B Cell Memory May Be Observed in Various Specific IgG Kinetics after Hematopoietic Stem Cell Transplantation

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Abstract: Background: Humoral memory and specific antibodies levels depend on kind of the antigen and individual immunofactors., The presence of IgM antibodies or fourfold rise in specific IgG level, are generally accepted as diagnostic in serology of acute viral infection. This basic model is not adequate for herpesvirome especially after hematopoietic stem cell transplantation (HSCT) due to continuous, usually multifocal antigenic stimulation, various donor serostatus, immunosuppresion and individual immunoreconstitution. Methods: A case-control study was conducted for identifying cases of Human herpesviruses (HHV) active infection (from 300 diagnosed immunocompromised patients) and evaluating historically associated humoral factors to look at outcomes. We enrolled only patients' data with meticulous differential diagnosis to exclude other causes, thereby observe pathways and temporal relationships, not statistical ones, usually collected in cohorts. Despite the small number such data collection and analysis avoids a number of biases and indicates cause and effect. Results. In the observational study retrospective analysis of data from 300 patients with clinical diagnosis of herpes simplex (HSV) and varicella zoster virus(VZV) reactivation showed a number of biases. In two well differentiated cases (confirmed by Tzanck test) with various disease and conditioning evolution of immunoparameters showed interesting pathway. Exponential decrease of specific IgG after HSCT precedes virus replication with cytopathic effect (shingles, VZV-encephalitis and HSV-induced mucositis). Minima (smallest IgG levels) before herpes virus reactivation were 234,23 mIU/ml and 94 RU/ml for VZV and HSV, respectively. This coincides with low CD4 titer, but without other infectious process. Other immune response parameters such as Treg, cytotoxic T cells, complement and total IgG level were quite the same as before the transplant procedure. Interestingly, the second wave of immunoreconstitution with anamnestic antibody response was not always observed. It coincided with prolonged herpes viral infection. Patient with lymphocyte depletion in conditioning showed earlier second wave of immunoreconstitution (6th vs 14th month). Conclusions: Typical for infancy kinetics of IgG level is unique after HSCT (the decline phase is first). Host-microbiome factors (i.e. HHV1-3 -serostatus) should be taken into account to predict risk of non-relapse mortality and survival after HSCT. The levels of specific antibodies help to predict the prognosis and improve the management. The lack of differentiation, assessor and confusing bias are the main obstacles in statistical HHV1-3 research. Such time-lapse case studies may be first to build evidence of a pathway and association between immunoparameters and HHV disease.

Keywords: Virome; Human herpes virus; Varicella Zoster Virus; Herpes Simplex Virus; Tzanck test; IgG protective level; hematopoietic stem cell transplantation; Hematopoietic Cell Transplant-Comorbidity Index

1. Introduction

Human herpesvirus (HHV) infections are widespread diseases that are benign in immunocompetent but may lead to severe complications and occasionally to death in severe immunocompromised patients. The decline of maternal anti-VZV antibodies in infants with

 doi:10.20944/preprints202312.1765.v1

chickenpox (the cut-off value was estimated 150 mIU/ml) was already described [1]. Further observations showed significant inverse correlation between varicella severity and the level of maternal anti-VZV IgG [2]. The same correlation was observed for neonatal herpes simplex virus (HSV) [3]. Analogous conclusions are being drawn from epidemiology i.e. discrepancy between high shedding rate among pregnant women with established HSV infection and low neonatal-transmission rate [4]. Successful prevention as the result of passive transfer of maternal antibodies (class IgG only) indicates that humoral response in HHV1-3 infection is pivotal. This simple model indicates the threshold of protection against primary infection, but for HHV-reactivation the protective level has not been estimated so far: insufficient data exist on the relationship between viral reactivations and immune reconstitution [5]. Furthermore, concentrations of specific IgG have been used to estimate immunity against Varicella Zoster Virus (VZV) in solid organ transplant (SOT) recipients only[6], but in hematopoietic stem cell transplantation (HSCT) it is more problematic: HHV1-3 serostatus is not evaluated and acyclovir -overused [7].Decrease of all immunoglobulin subclasses was observed in patients less than 100 days after HSCT and normalization after a year. Patients with low IgG levels showed a decreased survival rate and increased incidence of transplant-related mortality in spite of low HSCT-Comorbidity Index (HCT-CI) [8]. HCT-CI was shown to predict risk of no-relapse mortality (NRM) without analysis of virome risk factors (e.g. HHV1-3 serostatus).

This diversity of the microbiome according to the new definition (microbiota, viruses, free DNA and host elements, therapy), makes difficult prospective cohort studies, usually considering a small amount of factors. Furthermore, development of a clear case definition and patients selection are critical to effective investigation. The multifactorial area case study is a good tool for exploring rare diseases or when other study types are focused on a few of the most likely causative factors (here HCT-CI)[9]. In a cohort study a statistical association between a risk factor and a disease or condition was studied, contrary to causes of the disease or condition. This is because it may be difficult to determine the timing of the risk factor relative to the onset of the disease. On the contrary, case studies make possible to look at multiple risk factors at once, time-lapse analysis and cause-effect relationship (timing)(10,11). This is a completely different approach to research and data analysis, enabling translational medicine: converting discoveries all the way from bench to bedside

The aim of this study is to characterize pathway and relationship between anti-HHV IgG level and clinical presentation (disease severity) in patients with shift from latency to replication of HSV and VZV (i.e. HHV1/2 and 3 respectively).

2. Results

In the preliminary study, the analysis of the medical history of 300 patients diagnosed with HSV or VZV reactivation showed a number of biases (11). In most of patients with HSV and VZV disease the diagnosis was not confirmed and other diseases were not excluded (see section 4.1 and supplementary data). Therefore ascertainment and confusing bias were the most common in analysis and data collection [10].Multicenter cohort studies have the same disadvantages, as each researcher makes a diagnosis in a unique way without differential diagnosis and ruling out other causes e.g. drug allergy Graft-versus-host disease (GVHD) (see below). Finally two HSCT cases with well documented previous (primary) infection, later HHV reactivation with cytopathic effect i.e. positive Tzanck test (presence of acantholytic and multinuclear giant cells) were enrolled [11]. Time-lapse serological data collection was presented in Figure 1.

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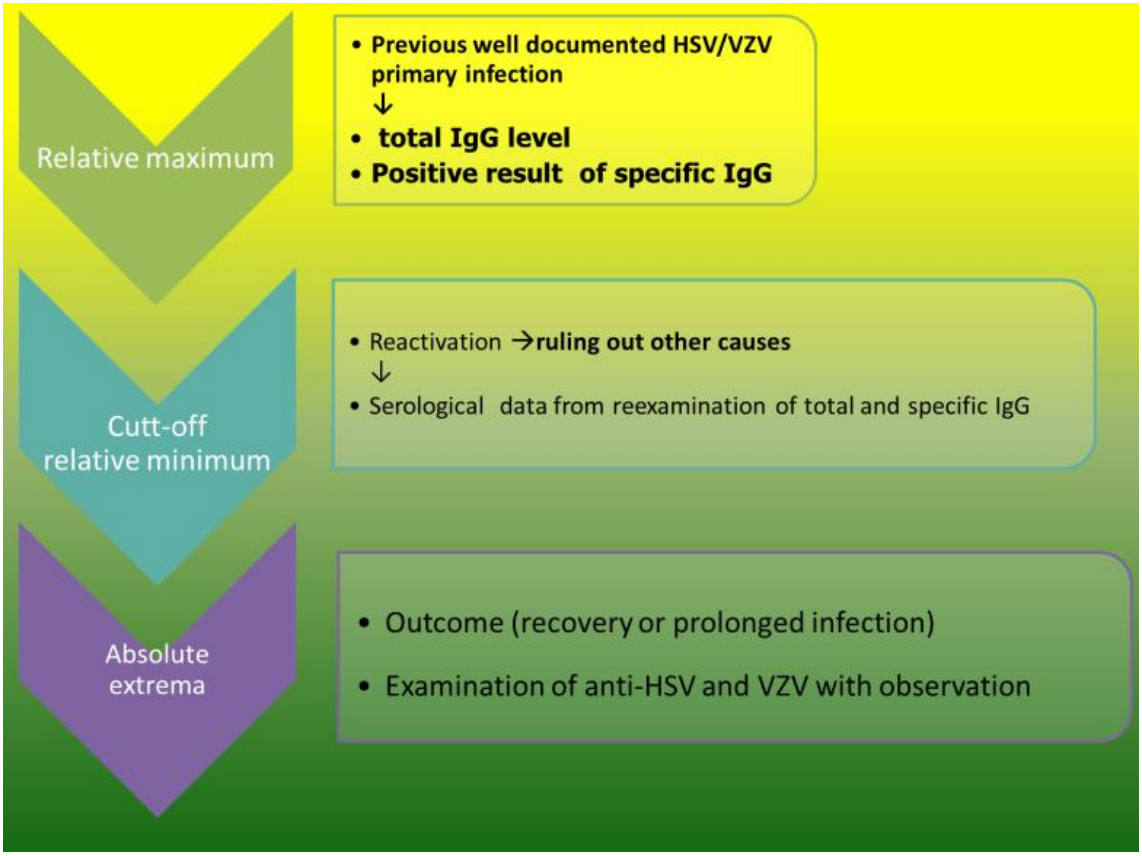

Figure 1. Flow chart of time-lapse observation and data collection. In our analysis the maxima and minima of IgG level, known collectively as extrema, are the largest and the smallest values of the specific IgG, either within a given range (e.g. 100 day after HSCT the local or relative extrema) or in the entire time (the absolute extrema).

Clinical immunoparameters, HSCT data from the two cases are summarized in Table 1.

Table 1. Clinical and immunological features of patients with HHV reactivation. The humoral immunodeficiency profile was observed. B cell and CD4-lymphopenia in patients P1 and P2 could be (in part) caused by cyclosporine A and poor second wave of immunoreconstitution after HSCT (Figure 2,3). The memory B cells (CD20 and CD27-positive) were reduced. Classical complement activation as non-invasive biomarkers of antibody-dependent cytotoxicity (ADCC) were not observed during VZV reactivation. Cellular compartment of specific immune response (HLA class I and CD8-dependent) was normal.

	P1		**P2**	
Gender	F		M	
Underlying disease	AML		SAA	
Conditioning	Bu-Cy		Flu-Cy-ATG	
Graft versus Host Stage Acute (chronic)	0 (1)		0 (0)	
Age	38		27	
	initial	**reactivation**	**initial**	**reactivation**
Complement C4 (mg/dL)	15.20	25.2	10.2	15.0
β2microglobulin (mg/l)	1.3	3.23	1.8	4.0
CsA (ng/ml)	ND*	137	ND	155
WBC (/ul)	3500	2530	7200	6500

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%lymphocytes (cells/µl)				
CD3	75.3(642)	78.1(↓444)	ND	60.0(752)
CD4	↓25.6(218)	↓↓15.1(86)	ND	↓25.3(317)
CD8	46.1(393)	64.3(366)	ND	28.6(359)
CD20	12.3(↓104)	↓↓2.5(14)	ND	↓5.2(65)
CD20+27+	↓1.2(10)	↓↓<0.5%*	ND	↓↓0.7(9)
CD20+23+	11.2 (96)	1.6 (9)	ND	3.5(44)
CD16+	8.1 (69)	8(45)	ND	7,7 (97)
CD16+56+	7.5 (64)	3.8 (21)	ND	6.7(84)

* ND –not done **below detection limit.

A patient 1 was admitted to our Center presenting severe herpesvirus complication. Earlier, the patient received hematopoietic stem cell transplantation from matched unrelated donor (10/10) with standard reduced intensity conditioning (RIC).

After full chimerism (i.e. +28 day), laboratory tests revealed the donor blood group without hemolytic anemia and the patient was in good health condition. The patient was discharged from hospital with follow-up visit every two months. GvHD prophylaxis consisted of cyclosporine A (CsA) with stable level and good efficacy (Table 1). Therefore, the patient did not receive prednisone for GVHD. One year after the transplant, the patient developed zoster (facial, then diffuse) complicated with viral encephalitis despite immediate administration of high dose of acyclovir. Local skin and mucous membrane blisters with stage 2-4 oral mucositis also developed, predominantly in the mouth and nose area .

Severe CD4 T and B lymphopenia was observed below 100 and 20 cells/µl, respectively However, other signs of abnormal CD8+ cytotoxic T cell level and Tc-mediated immunity were not observed. The VZV and HSV reactivation coincided with low CD4 Th and CD20 B cells titer (Table 1), but CMV and EBV PCR results were negative. Retrospective analysis of serum samples showed specific anti-HSV and anti-VZV IgG level fluctuation. Total IgG level was stable (about 1000mg/dl) (Figure 2).

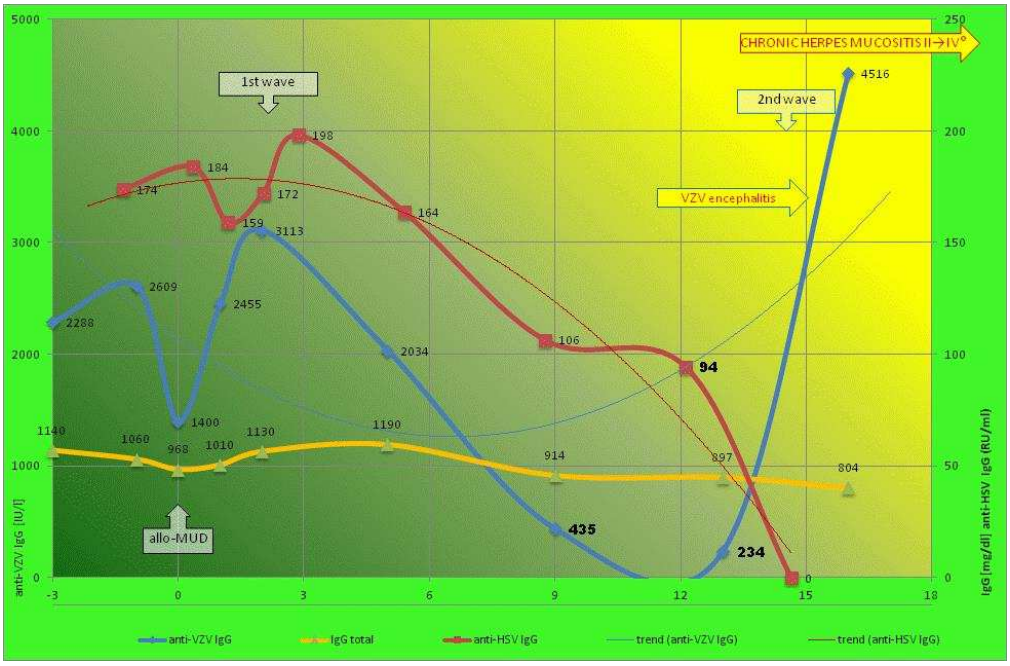

Figure 2. Patient 1 (P1) with ineffective acyclovir therapy and late second wave immunoreconstitution within a period of 18 months. Levels of specific antibodies were presented in logarithmic scale. Trend lines (two different directions) reflect different prognosis.

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Exponential kinetics of specific anti-VZV IgG level (exponential decrease and increase within eighteen-month observation) was presented with parabolic trend-line. Contrary to anti-VZV, prolonged herpes mucositis was observed with significant (also exponential) decrease of anti-HSV. Despite temporary increase in first months, the trend line oh anti-HSV was inverted hyperbola (exponential function type a^x, where $a<1$) that coincides with late onset and increase of the severity of prolonged mucositis (Figure 2). Contrary to exponential change of HHV-specific IgG, total IgG level was stable without significant extrema. First and second wave immunoreconstitution was observed only for VZV-specific IgG, contrary to anti-HSV (only first) and total IgG (no significant peak value).

The lowest IgG level before HHV reactivation was 234,23 mIU/ml(IU/L) and 94 RU/ml for VZV and HSV, respectively. However, the total IgG level was within reference range, but serum level of -β2-microglubulin was high (Table 1). Host-VZV dynamics showed acute and resolving infection i.e. setting where VZV initiated acute infection that then rapidly demonstrated very high anti-VZV IgG level (till 4515IU/L) with shift to the latency. The low and decreased level of anti-HSV IgG corresponded with prolonged oral mucositis (II→IV^0). No complement abnormality was observed nor the sign of GvHD at +100 day and later (Table1). As the observed entire abnormality of skin was explained unequivocally by a non-GVHD documented cause, the skin eruption was not included in calculation of the global severity [12]. Contrary to VZV specific antibody, vaccine-induced anti-HBs was high and total IgG levels were stable (Table 2).

Table 2. Humoral immunoparameters and class switch recombination (CSR) for Human herpes virome-specific antibody. The stable total class IgG and vaccine-induced anti hepatitis B surface antigen (HBsAg) antibody levels are presented for comparison. Noteworthy, IgM antibody response did not proceed four phases of IgG after a foreign antigen challenge.

*Noteworthy, for patient P1 different kinetic was observed i.e. without second wave of immunoreconstitution for specific immunity and significant drop of anti-HSV IgG to zero (see Figure 2), avidity could not be determined.

Level	P1	P2
IgG total	804-1190	332-980
IgG anti-EBV (Ru/ml)	>200	>200
IgG anti-CMV (RU/ml)	>200	>200
IgG anti-HSV* (RU/ml)	178→0	7,73→124
(avidity)	(40→NA)	(ND)
IgG anti-VZV (IU/l)	234→4515	32→1295
(avidity)	(78)	(72)
IgG anti-HbsAg**	660	160

**other serological markers of HBV as well as HIV, HCV were negative. NA-not applicable, ND – not done.

Patient No2 developed severe oral mucositis and diffuse zoster after HSCT, but in the second and third month after HSTCT, respectively. The patient's initial serological analysis showed high HHV-specific IgG levels before HSCT. Under the influence of immunosuppressive therapy (with ATG –Table 1) the coincidence of VZV reactivation, progressive oral mucositis and decrease of HSV- and VZV-specific IgG level were observed earlier. The inverse correlations between the severity and serological parameters were found during progression as well as oral/cutaneous healing (U-shaped parabolas, Figure 3). Total IgG levels were not stable (between 300 and 1000mg/dl) with significant peak values (extrema).

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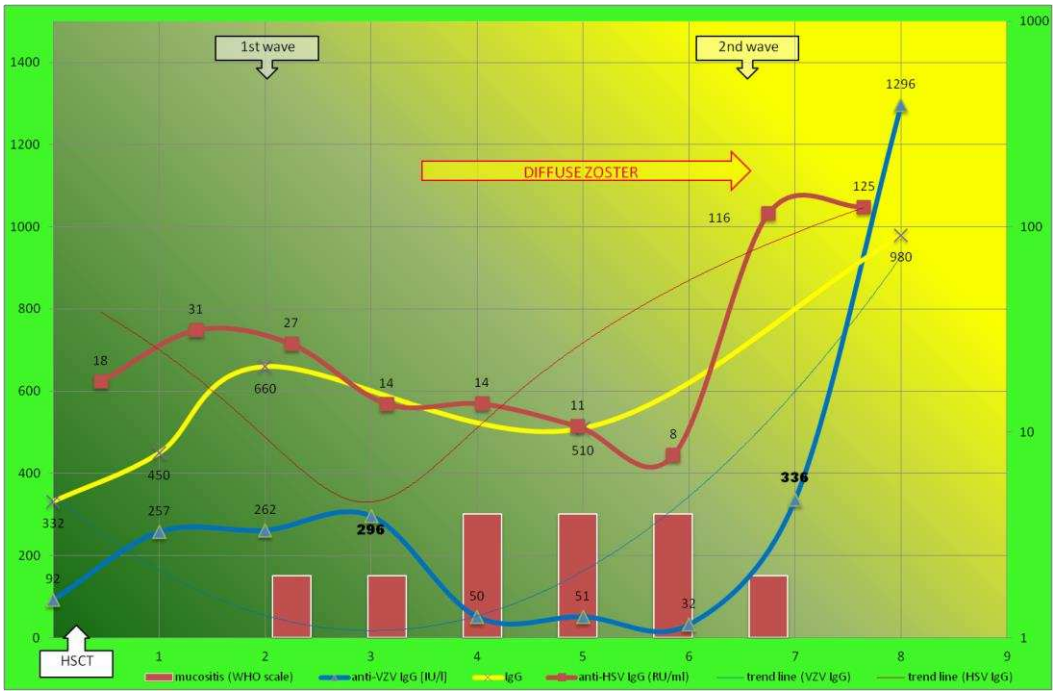

Figure 3. The second Patient (P2) after reduced intensity conditioning (RIC) with antithymocyte globulin (ATG) within 9-month follow-up.P2 after HSCT showed parallel distribution of HSV and VZV specific IgG (approximately parallel U-shaped parabolas). Levels of specific antibodies were presented in logarithmic scale (exponentially).

The first wave of immunoreconstitution showed short-term immunoprotection. The second wave gave significant (also exponential and more than 10-fold) specific antibody increase with long-term protection. Contrary to patient 1 the second wave of immunoreconstitution was observed earlier (about +6 month after HSCT) and for both –HSV- and VZV-specific antibodies, but less intensive second wave corresponded with prolonged zoster (Figure 3). The increase of anti-HSV IgG preceded oral mucosal barrier healing (after +7 month) The first and second waves (to a lesser extent and not exponentially) were observed in extrema of total IgG level.

3. Discussion

3.1. Technical aspect and clinical model

In HSCT contrary to primary infectious process, when the virus replication is local (in portal of entry), the HHV reactivation is systemic [13]. The exposure type: local or systemic may be important as our cut-off level for VZV reactivation is higher than described previously (1,2). The subtle difference is important when we look at immunoprophylactic (vaccination) or immunotherapeutic regimen.

Secondly, the methods of analysis and cut-off titer definition are different. Commercial assays of antibodies against HSV are not validated (4). Furthermore, discrepancy between the protective level (i.e. 150 IU/L) (1,2) and cut-off value reported to facilitate testing by manufacturers (e.g. 100 IU/L for Euroimmun) indicate that in laboratory practice determining the cut-off values for disease vs. healthy is done empirically. It is not protective level.

Thirdly, two images from the same patient might look very different)[14]. Since molecular testing is rarely used for VZVHHV1-3 infections, even in immunocompromised patients, availability is limited (15). Here VZVHHV1-3 reactivation was firstly diagnosis of exclusion, secondly – by acantholytic and multinucleated giant cells in the image (Tzanck test)(15). Due to the rapid turnaround time and correlation with clinical symptoms, serologic methods are preferable for VZV1-3 contrary to viral isolation [15]. Our time-lapse observation and immunomonitoring of transplant patients are valuable for assessing the immune status (immunoreconstitution) and reactivation risk.

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3.2. Immunomodulation and host-virus interaction in transplant patient

Herpesviruses such as HHV1-3, through the use of different genetic programs (latency versus lytic replication) are conditionally cytopathic and only cause tissue damage upon entry into the lytic cycle. For latent VZVHHV1-3 replication the immunological ignorance is the first strategy to avoid immune response (HSV and VZV preferentially infect immune-privileged sites). Furthermore, a prospective study of 281 allogenic HSCT recipients found a 3-year cumulative incidence of HHV-encephalitis due to herpes virus infections of 6.3% [16].

Other immunomodulatory mechanisms (e.g. down regulation of MHC class I or loss effector functions by CD8 T cells) were not observed in the our patients (Table 1). Noteworthy, β2-microglobulin (invariant chain of HLA class I) level was high and peripheral blood lymphocyte subset (percentages) showed normal or very high cytotoxic T cells (CD8+) (Table 1) as compared with healthy donors (i.e. 27.64–30.66%) [17].It turns out that β_2M plays a role in the humoral response. Patients lacking its expression show humoral disorders with low IgG and severe skin disease [18].

Humoral counterpart is critical, as presented previously for VZV (1,2), HSV (4) and CMV (13). On the contrary, decrease in Th cells is the primary cause of the impaired humoral response in older adults and low level of CD4 T helper cells corresponded in our observation with the decrease of specific antibody synthesis (Table 1), but major immunomarkers did not reflect specific response formation. Although the transplantation procedure itself (conditioning, immunosuppression) is standardized, the course and immunological reconstitution in patient 1 was far from the general scheme, since anti-HSV was not seen in the second wave of reconstruction (Figure 2). The vigorous immune response to VZV (virus close to HSV) was not protective against HSV and severe mucositis in spite of cross-reactive antibodies between HSV-1 gB and VZV gp-II [19].

Interestingly, the clinical symptoms were observed after rapid decrease of specific IgG. Unfortunately, presence of fourfold rise in specific IgG level, are generally accepted as diagnostic in serology of acute viral infection (15). In our patients the increase of specific IgG and its kinetics shows progress from a primary immune to the memory state as described elsewhere [20]Therefore observation of host IgG dynamics may be hallmark of HHVs shift from latency to replication as well as preclinical initial stage of zoster or mucositis.

3.3. Host-virus dynamics

Classical Primary Antibody Response proceeds in the following four phases after a foreign antigen challenge (15):

1. Lag phase—very low/ no antibody is detectable.
2. Log phase—the antibody titer increases logarithmically.
3. Plateau phase—the antibody titer stabilizes.
4. Decline phase—the antibody is catabolized.

Secondary (Anamnestic) response exhibits the same four phases of primary response, but after HSCT this scenario, especially for herpesvirome, is not adequate (reexposure vs continuous exposure). At least the order of events seems reverse, similar to infancy (1), when the first phase is a decline phase with catabolism of residual pretransplant (as maternal) IgG (Figure 2,3) (13). The first after HSCT phase (decline phase) in our patients was also logarithmic/exponential.

Our observation shows two different known virus-host dynamics [21]. As presented in figure 2, VZV sets up acute infection that is then rapidly reduced (switched off to the latency cycle). On the contrary, HSV induces long-term lytic replication with clinical sign of cytopathic effect in mucosa with many erosions, ulcers and WHO stage 3/4 mucositis in patient 1. The former manner is characterized in typical parabolic vertical trend line (Figure 3 and line for VZV in Figure 2), but the latter – in inverted hyperbola (line for anti HSV in Figure 2). The delicate balance between the two host-virus dynamics is not visible in standard laboratory and clinical practice after HSCT. Interestingly, we observed two different virus-host dynamics of VZV in one patient (P1) after HSCT therefore under influence the same therapeutic regimen (Figure 2). VZV in patient 2 (contrary to patient 1) immunoreconstitution of HSV- and VZV-specific IgG developed in parallel manner. In HSCT the presence of CMV-specific IgG (in donor) is a known, excellent predictive indicator of the

 doi:10.20944/preprints202312.1765.v1

likelihood of significant posttransplant infection . There is no such observation for HHV1-3. Our patient with well documented past history of herpes or chickenpox indicates the crucial role of anti-HHV1-3 IgG against reactivation (Figure 2 and 3). Unfortunately, according to current practice the donors are screened for CMV only. Therefore, two different antibody kinetics of VZV in patient 1 may indicate different donor serostatus: for VZV and HSV1/2, respectively.

Specific IgG against glycoprotein C of HHV1, tested here, play a crucial role in infectious process just in a different way. Glycoprotein C (GC) of HSVplays a principal role in the adsorption of virus to cells [22] and blocks complement cascade by the C3b binding, inhibiting interaction with C5 and properdin and therefore formation of C5 convertase [23]. Passive transfer of specific IgG against glycoprotein C did not protect complement intact mice against HSV-1 [24]and the reduction of titer was anti-GC-dependent (27). In fact, the opposite phenomenon was observed in our patient: decrease of GC-specific antibody caused fast HSVreplication and oral mucositis was observed before zoster (Figure 2). Therefore, the protective level for HSV-1 may be higher than VZV or other herpesviruses. Low concentration of anti-VZV or GC-specific IgG is required for opsonization (and ADCC), but in higher titer – blocking of adsorption and C3b binding by GC .

3.3. Humoral compartment in secondary immunodeficiency after HSCT, immunomodulation by conditioning

The percentages of CD8 cells were normal, contrary to Th (CD4) and B (CD20) cells (Table 1). Good model for humoral immune reconstitution post-HSCT is allogenic stem cell transplantation in common variable immunodeficiency (CVID). As shown in one study, the percentage of patients withdrawn from immunoglobulin replacement was 25, 40 and 60 percent in the first, second or third year after HSCT, respectively [25].Our observation coincides with the data from these previous studies. In our patients deep decrease of specific immunoglobulin production was also between 6 months and less than 17 months (Figure 2,3). Low immunoglobulin levels are associated with decreased survival as described previously [26]. On the contrary, in P1 patient total IgG level was stable (about 1000mg/dl) and only in P2 the two wave of humoral immunoreconstitution was weakly observed in total IgG.

The explanation of the data may be different time of reconstitution of several antigen-specific Th and B cells or secretion of IgG by several plasma cells (Table 2). Second issue was ATG use in patient 2. ATG directly affects not only T cells (especially Th with low CD4/8), but also B lymphocytes , so humoral immunoreconstruction occurs in synchronous way and two maxima of total IgG level are observed (Figure 3). The MAC regimen and cytostatic cause inhibition of the cell cycle arrest at different stages, therefore the lymphocyte renewal with selection of individual clones start again differently, especially in second wave (Figure 2) with various profile of memory B cells.

3.1.2. B cells memory

Memory B cells (MBC) are generated during primary immune response. MBC do not produce antibodies, unless re-exposure to antigen drives their differentiation into antibody producing plasma cells. Interestingly, our patient P2 had a high affinity for antigen, contrary to low level CD20CD27 B cells i.e. canonical surface phenotype of MBC (Table 1,2). The same temporally protective increase of specific anti-VZV IgG and without “memory” was observed in first waves in patients after HSCT. The low CD20+27+ memory B cells may be in part explanation of the phenomena. Furthermore, the clinical model and two-peaks IgG dynamics observed here after HSCT correspond with last finding of two phases of the acquisition of B cell memory [27]. Oral mucositis and HSV coexistence is a well described phenomenon after HSCT [28,29]: following HSCT anti-HSV is a predictor of ulcerations on oral mucosa [30,31].

In SOT, the memory B lymphocytes (MBC) and long-lived plasma cells (PC) are preserved, in HSCT they are not. These cells are usually depleted (with antithymocyte globulin (ATG) or myeloablative conditioning) and replaced by donor-derived with full chimerism (13). Noteworthy, in our patients the temporary increase of IgG corresponded with low MBC level and comparably higher level of active B cells (i.e. CD20+CD23+)(Table 1)

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After first step of B cell differentiation short-lived plasma cells may be produced but lifespans are generally limited to the course of the infection (27). CD20 and CD23-positive B cells - demonstrated in our patients are derived from germinal center B-cells with activatory signature. Interestingly, subsequent recall responses to HHV antigens during virus replication caused, significant increase of specific IgG level, low level of memory B cells: probably differentiating into long- or short-lived plasma cells (27).

3.1.4. Two signal and IgG increase

In vivo viral and membrane-associated antigens arrive in the lymphatic organ via subcapsular sinuses. The movement and concentration of membrane IgG (regulation of cytoskeleton) are observed after stimulation by corpuscular antigens and B cell response to soluble or monovalent antigens and require more co-receptors [32]. Noteworthy, HHVs are the source of double signal: specific (by surface IgG) and non-specific –by pattern recognition receptor e.g. Toll-Like receptor (TLR). As described previously for CMV, TLR signal prompts fast IgG response without IgM (13). Furthermore, study with two signal immunizations (specific and TLR9 i.e. by protein antigens and CpG) resulted in short-lived plasma cell generation and high levels of antibody that failed to affinity maturation [33](graphical abstract). It is consistent with low MBC (Table 1) and temporary increase of HSV- and VZV-specific IgG in our patients (Figure 1,2). Viral genomic DNA is known ligand for TLR9, however, for HSV and VZV which has been reported recently [34,35]. It corresponds with the latest observation of immunodeficiency with vigorous VZV-susceptibility and TLR-3 variant/mutation [36,37].

Our retrospective analysis, has several limitations such as a small group. On the other hand, all previous studies assessing the risk of HSV/VZV reactivation assume an earlier history of the primary infection. This is not clear today. Vaccination against VZV is used more and more often, which makes differentiation difficult (as in the presented infant situation (1,2). In retrospective analysis strict and comparable observation is difficult. Therefore, a very rigorous differential diagnosis and selection, impossible in cohort studies, was assumed. Sometimes isolated results were reported in individual patients, with no adequate equivalents in other patients (for example HSV avidity)(13). On the other hand, the extrema i.e. the largest and smallest value of the IgG level, either within a given range (the local or relative extrema in the first wave of immunoreconstitution), or on the entire time (the absolute extrema) is difficult for observation in cohort studies. Time-lapse analysis and case-control study is better to discover any change that may set patient apart from others. The model adopted here can be a starting point for further, prospective, long-term observations. Specific IgG evolution in parabolic or hyperbolic manner probably corresponds with various outcomes (Figure 2, graphical abstract). HHV reactivation and kinetics of IgG level typical for primary immune response are unique after HSCT as well as neonatal HHV infection . Unfortunately most studies are focused on the optimal duration of antiviral prophylaxis (7,[38]. There is variability in clinical practice across transplant centers, but preventive antiviral therapy showed low efficacy in our patients and second wave of immunoreconstitution of specific humoral immune response played a significant role. Interestingly, cumulative incidence of VZV disease after HSCT was higher at 2 year after HSCT with non-significant influence of conditioning (RIC/MAC) and ATG use (38). It is generally in line with our observation for VZV (noteworthy P1 and P2 developed VZV reactivation), but cumulative incidence does not show differences and timeline, as mentioned above (9). However, in this cohort study (141 patients), only six had VZV disease, and the diagnosis was made without differentiation. Therefore, this study was not free from the described above bias (10), since microbiological, pathological, and/or serological confirmation was not performed (38).

Studies on HSV have not been conducted so far, and our observation shows a significant difference in conditioning (RIC+ATG vs MAC) (Figure 2 and 3).

 doi:10.20944/preprints202312.1765.v1

4. Materials and Methods

4.1. Material

The observational study comprised the history of 300 patients with HHV1-3 reactivation. Only patients diagnosed with humoral immunodeficiency with well documented previous HSV or VZV primary infection in an interview were included. The patients were a heterogeneous group of various primary or secondary immunodeficiencies (detailed in supplementary material). Simple statistical analysis could not be used due to a number of biases. Firstly, because people were not randomized to outcome group and dissimilar in their demographic and clinical characteristics. Therefore, the study was prone to confounding. .

Secondly, the symptoms of herpes zoster and eczema herpetiformis often overlap with graft-versus host, autoimmunity, drug allergy and other infectious complications in immunocompromised patients (HHV1-3 reactivation was a diagnosis of exclusion). Thus, the data from patients with mis-differentiation, without Tzanck test for confirmation and excluding other causes, were not included in the study. Consequently, most of patients were disqualified (supplementary material).

4.2. Time-lapse data collection

Our previous study adopted the Pinquier's model (1) for CMV reactivation during infancy and introduced it during cytomegalovirus reactivation in transplant patients after HSCT (13). The current study investigated the role of herpes simplex 1/2 and varicella-zoster-specific IgG (i.e. HSV and VZV, respectively) The flow chart of the systematic clinical observation and data collection before and after transplantation was presented in Figure 1.

4.3. Methods

In our Center serum samples were collected during every clinical visit. Blood samples (0.5 ml) were collected in a dry tube and centrifuged for 10 to 15 min at 3,000 rpm. After centrifugation, the serum was extracted and stored at 20°C. The specific VZVanti-HHV antibodies were tested with EUROIMMUN ELISA kits. Recombinant glycoprotein C1(GC) HHV1 and virus of the „VZ-10" strain (highly purified cell lysate from MCR-5 cells infected with varicella zoster) were used as an antigen. The data were expressed in accordance with the international reference preparation W1044 (WHO) as IU/L (i.e. mIU/ml as described by previously (1,2). Neither of the reference standards were defined completely by the manufacturer for HSV (4) therefore, the data are expressed in RU/ml as described by the manufacturer. Positive results are >110 IU/l and >22RU/ml, negative <80 IU/l and <16IU/ml for anti-VZV and anti-HSV IgG respectively. According to the manufacturer's description, results between these values were considered as equivocal. The affinity IgG was presented as relative avidity index (RAI) as described by manufacturer and expressed in %.

Leukocyte count analyses were done by the Sysmex Automated Hematology System. Flow cytometry was performed using a FACS Calibur flow cytometer (Becton Dickinson). According to standard operating procedure in our Center, the after CD45-based gating of lymphocyte, lymphocyte subsets were calculated by multiplying the percentage of these lymphocyte subpopulations by lymphocyte counts (13).

Patients monitored in our outpatient clinic were systematically serologically tested for IgG level against HHV, i.e. CMV,EBV, VZV and HSV. Cut-off value was estimated when the patients developed reactivation.

5. Conclusions

Summing up these observations, the logarithmic/exponential decrease of specific IgG should be considered as a hallmark of high likelihood of virome dysbiosis and opportunistic HHV1-3 infection. These findings have implications for vaccination and adoptive immunotherapy or passive immunization strategies in transplant patients. There is variability in clinical practice across transplant centers, but acyclovir therapy showed low efficacy in our patient until second wave of

immunoreconstitution of specific IgG with crucial role. Furthermore, the cut-off level and threshold of protection against HHV reactivation is higher than in primary infection (1,2). Therefore, 239 IU/L or 100 RU/ml indicates the point of initiation of active or passive immunization for VZV and HSV, respectively.

Although current EBMT, WMDA standard requires monitoring of several infections (e.g. HIV, HTLV1/2, HBV, HCV, CMV, Treponema) the donor immunostatus against all HHVs and Tzanck test should be introduced in transplant practice. The lack of differentiation and bias are the main obstacles in statistical HHV1-3 research. Such time-lapse case studies may be first to build evidence of a pathway and association between immunoparameters and HHV disease. This approach brings translational medicine closer to experimental research, where each patient case is a unique, yet strictly and carefully described experiment of nature. Quite opposite , cohort studies are highly simplified operating only a few parameters. (for example HCT-CI).

HCT-CI and calculation of the global severity should be extended to include such important parameters as virome homeostasis. HHV1-3 reactivation after immunoablative RIC (with ATG) and MAC seems to be just as likely, but the timing of manifestation and outcome are different and may be an introduction to further research in the future.

Author Contributions: P.Z. collected and analyzed data, wrote, reviewed, and revised the manuscript. A.G. at the Institute of Immunology provided funding for the work, read and revised the manuscript.

Funding: This work was supported by the statutory activity of Hirszfeld Institute, Lower Silesian Center and from private funds of the first author.

Institutional Review Board Statement: Not applicable: retrospective nature of the study.

Informed Consent Statement: Patient consent was waived due to retrospective analysis of 300 patient's history with anonymization of data. Written and informed contents was received from P1 and P2 for the publication of the laboratory results.

Acknowledgments: The author would like to thank Koćwin E. and Bocheńska J. for the serological test. Many thanks to Janina Grycewicz for the linguistic proofreading.

Conflicts of Interest: The author declares no conflict of interest.

Abbreviations

SID acquired/secondary immunodeficiency
ATG, antithymocyte globulin
CMV cytomegalovirus
EBV -Epstein–Barr virus
GC -glycoprotein C1
Conditioning: myeloablative (MAC) or reduced intensity (RIC)
GvHd –graft versus host disease;
HBV -Hepatitis B virus
HBs -surface Hepatitis B antigen
HCV Hepatitis C virus
HHV - Human Herpesvirus;
HSCT - hematopoietic stem cell transplantation
HSV -Herpes Simplex Virus
IgGSD -IgG subclass deficiency
PC -long-lived plasma cells
MBC -the memory B lymphocytes ()
PAMPs pathogen-associated molecular patterns
PAD -primary antibody deficiency
RAI -relative avidity index
SIgAD -selective immunoglobulin A deficiency
SOT -solid organ transplant
TLR -Toll-Like receptor

THI -transient hypogammaglobulinemia of infancy
VZV = Varicella Zoster Virus;
WMDA -WORLD MARROW DONOR ASSOCIATION
B2M -β2-microglubulin
Drug i.e. MEL, indicates melphalan, BU, busulfan; Flu, fludarabine CY cyclophosphamide

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