Table 2 Clinical background and laboratory data of patients with hydroa vacciniforme (HV) and/or hypersensitivity to mosquito bites (HMB)

	cHV	sHV	HMB only	HMB + HV
Cases (50 in total), n	23	12	9	6
Sex (male/female)	13/10	5/7	6/3	2/4
Median age at onset age (years)	5	8	8	3·5
Clinical symptoms, n				
Fever	0	8	9	5
Lymphadenopathy	0	2	7	1
Hepatosplenomegaly	0	4	3	4
Aphthous stomatitis/gingivitis	6	5	0	0
Conjunctivitis/iritis	4	1	0	0
NK lymphocytes > 30%, n/N	0/9	2/7	6/7	6/6
γδT cells ≥ 5%, n/N	7/8	3/3	0/1	ND
Cases followed up (30 in total), n	11	8	6	5
Follow-up time (years), median (range)	8 (1–26)	7 (4–9)	3 (1–5)	12 (1–19)
Laboratory data (mean ± SD)				
White blood cells ($\times 10^3$ per μL)	7·14 ± 2·69	5·27 ± 2·34	5·87 ± 2·17	5·72 ± 1·76
Haemoglobin (g dL^{-1})	12·7 ± 0·7	11·8 ± 1·8	12·4 ± 1·5	13·3 ± 1·3
Platelets ($\times 10^4$ per μL)	28·0 ± 6·3	20·5 ± 8·2	13·0 ± 5·6*	27·0 ± 6·9
Lactate dehydrogenase (U L^{-1})	258·6 ± 79·7	289·0 ± 201·8	455·5 ± 176·8*	265·3 ± 91·2
Aspartate transaminase (U L^{-1})	26·6 ± 10·1	32·5 ± 12·9	74·8 ± 78·0	53·0 ± 51·9
Alanine transaminase (U L^{-1})	19·4 ± 12·7	31·9 ± 21·2	117·0 ± 145·0	53·5 ± 65·8
NK lymphocytes > 30%, n/N	0/8	2/6	6/6	5/5
γδT cells ≥ 5%, n/N	4/5	3/3	0/1	ND
EBV antibody titre[a] (mean ± SD)				
VCA IgG	120 ± 97·4	80 ± 432·0	40 ± 1031·6	640 ± 1210·1*
EA IgG	0 ± 5·3	10 ± 70·3	5 ± 5·5	20 ± 27·9*
EBNA	15 ± 101·9	15 ± 25·6	15 ± 71·1	10 ± 8·4
EBV DNA load (PBMCs), $\times 10^4$ copies μg^{-1} DNA	9·72 ± 22·23	5·61 ± 3·76	3·64 ± 5·88	5·15 ± 5·41
Mortality rate, n (%)	0 (0)	3 (38)	2 (33)	2 (40)
Complications	–	HPS (1), myocarditis (2), gastrointestinal bleeding (2)	Unknown	HPS (1)
Cases examined by immunostaining (19 in total)	6	5	4	4
Dominant lymphocyte subset in skin lesion	CD3ε+CD56− (6/6)	CD3ε+CD56− (5/5)	CD3ε+CD56+ (4/4)	CD3ε+CD56− (1/1 HV and 1/3 HMB); CD3ε+CD56+ (2/3 HMB)

cHV, classical HV; sHV, systemic HV; NK, natural killer; ND, not determined; EBV, Epstein–Barr virus; VCA, viral capsid antigen; EA, early antigen; EBNA, EBV nuclear antigen; PBMC, peripheral blood mononuclear cell; HPS, haemophagocytic syndrome. *$P < 0·05$ vs. other subgroups. [a]Fluorescent antibody method.

4-year-old girl with cHV, progressed to sHV in the follow-up period due to an episode of fever associated with rash, and underwent haematopoietic stem-cell transplantation (HSCT).[15] The patient had no BZLF1 mRNA expression in the skin lesions, and 83·6% of the γδT-cell fraction was positive for EBV infection.

Of 19 patients with sHV, HMB and HMB + HV, seven (37%) died during the follow-up period, including three of eight patients (38%) with sHV, two of six patients (33%) with HMB only and two of five patients (40%) with HMB + HV. The main cause of death was related to HSCT in at least two patients, and was multiorgan failure in another three.

Fatalities were observed only in groups with systemic symptoms (i.e. sHV, HMB only and HMB + HV), and not in the cHV group (Fig. 1). The log-rank test demonstrated a poor prognosis in patients with sHV and HMB only, compared with those with cHV ($P = 0·016$ and $P = 0·015$, respectively). Patients with cHV were distinct from the other three groups in terms of prognosis ($P = 0·026$), but no significant difference in prognosis was observed between the HV group and the HMB group ($P = 0·29$).

The number of fatal cases increased gradually over 10 years and did not plateau, except in patients with cHV, who showed no fatalities. The cumulative survival rates reached below 50% in 4 years in patients with HMB only, 9 years in sHV and 14 years in HMB + HV. There was a significant difference in cumulative survival between the groups with HMB only and sHV ($P = 0·031$).

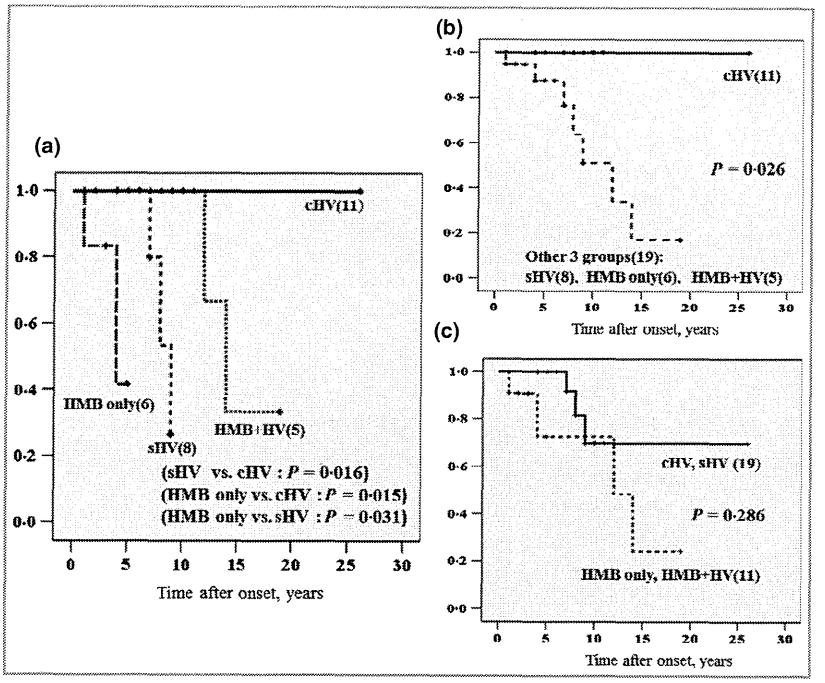

Fig 1. *Survival time of the four groups by the* Kaplan–Meier method. All patients with classical hydroa vacciniforme (cHV) survived during the observation period. (a, b) Patients with cHV showed significantly better prognosis than patients with systemic HV (sHV) or only hypersensitivity to mosquito bites (HMB) by log-rank test (P = 0·016 and P = 0·015, respectively), and better prognosis than the other three groups (P = 0·026). (c) There was no significant difference in the survival time between patients with only HV or HV-like eruptions and patients with HMB (P = 0·286).

Blood test results for each group

Routine laboratory test results on diagnosis showed no significant differences between the groups in white blood cell count, haemoglobin or aspartate aminotransferase (AST) (Table 2). However, in the HMB-only group, three of six patients (50%) had platelet counts below 15×10^4 cells μL^{-1}, and they had higher serum levels of lactate dehydrogenase than the cHV or sHV subgroups (both $P < 0{\cdot}05$). Patients with sHV showed elevated levels of AST, alanine transaminase and lactate dehydrogenase to varying degrees, and haematological abnormalities such as leucopenia and thrombocytopenia, suggestive of haemophagocytic syndrome.

Lymphocyte subsets in PBMCs, taken together with the additional data described in our previous research,[3] revealed that 10 of 11 patients (91%) with HV-like cutaneous lesions, whether in cHV or sHV, showed elevated γδT-cell percentages > 5% (range 2·2–25%), while 12 of 13 patients (92%) with HMB only or HMB + HV showed NK-cell lymphocytosis of > 30% of PBMCs (range 2–85%).

Antibody titres against EBV-related antigens, as determined by immunofluorescence study, demonstrated no differences in EBV nuclear antigen. However, IgG titres against anti-early antigen and IgG-class antiviral capsid antigen were slightly higher in the HMB + HV subgroup than in the cHV and sHV groups ($P < 0{\cdot}05$), and in the cHV and HMB-only groups ($P < 0{\cdot}05$).

The EBV DNA load in PBMCs, as determined by qRT-PCR, was < 100 copies μg^{-1} DNA in healthy individuals, but higher than the reference values in all 26 samples from our patients. The range was 770–720 000 copies μg^{-1} DNA, with a mean value of 67 420 ± 140 224 copies μg^{-1} DNA. There were no statistically significant differences among the patient groups (Table 2).

Histopathological examinations

The percentages of $EBER^+$ cells varied, even in the same disease group, ranging from 1% to 25% of the infiltrating mononuclear cells in all but two of 21 patients analysed. In the two exceptional cases, > 25% $EBER^+$ cells were observed in the infiltrates in one patient with sHV and one with cHV. No difference was observed in the number of $EBER^+$ cells among the cHV, sHV and HMB-only groups. The number of samples in the HMB + HV group was insufficient for statistical analysis.

In agreement with our previous report,[3] $CD3\varepsilon^+$ and $CD56^-$ T cells were predominantly infiltrating in HV lesions, without correlation to the severity of the cutaneous lesions. However, in HMB lesions, in addition to $CD3\varepsilon^+$ and $CD56^-$ T cells, many $CD56^+$ cells suggestive of NK cells were present in six of the seven cases examined. The numbers of reactive T or NK cells negative for EBER were usually larger than the numbers of $EBER^+$ cells.

Onset age as a prognostic factor

We also evaluated prognostic factors by univariate analysis (Table 3). We analysed the 30 patients who were available for follow-up, consisting of 11 with cHV, eight with sHV, six with HMB only and five with HMB + HV. Onset age over 9 years was significantly correlated with mortality by univariate analysis ($P < 0{\cdot}001$), and this association was stronger than that between mortality and onset age over 8 years ($P = 0{\cdot}026$). No significant correlation was observed regarding sex; clinical symptoms such as fever, splenomegaly or lymphadenopathy; routine blood test results or HSCT treatment.

Table 3 Prognostic factors for hydroa vacciniforme (HV) and hypersensitivity to mosquito bites (HMB)

Factor	Alive	Dead	P-value[a]
Onset (years), mean ± SD	5·6 ± 3·1	12·6 ± 8·6	0·077
Onset ≥ 9/< 9 years (total)	1/22	5/2	< 0·001
Onset ≥ 9/< 9 years (cHV + sHV)	1/15	3/0	0·004
Onset ≥ 9/< 9 years (excluding cHV)	1/11	5/2	0·0095
Onset ≥ 8/< 8 years (total)	5/18	5/2	0·026
Male/female	11/12	2/4	0·663
Fever, yes/no	9/14	5/2	0·204
Splenomegaly, yes/no	2/20	3/4	0·075
Lymphadenoma, yes/no	3/20	3/4	0·12
Laboratory data (mean ± SD)			
White blood cells ($\times 10^3$ per μL)	5·57 ± 1·97	6·20 ± 2·02	0·85
Haemoglobin (g dL^{-1})	12·3 ± 1·2	13·2 ± 1·8	0·377
Platelets ($\times 10^4$ per μL)	24·0 ± 9·1	17·8 ± 5·1	0·071
Lactate dehydrogenase (U L^{-1})	299·4 ± 128·8	344 ± 254·2	0·694
Aspartate transaminase (U L^{-1})	43·2 ± 45·4	36·5 ± 33·2	0·232
Alanine transaminase (U L^{-1})	47·7 ± 83·1	47·7 ± 46·7	0·414
DNA load ($\times 10^4$ copies μg^{-1} DNA)	7·34 ± 1·55	4·18 ± 3·61	0·753
VCA IgG (titre)	80 ± 282·3	160 ± 1331·8	0·226
VCA IgM (titre)	10 ± 5·1	5 ± 5·5	0·932
EA IgG (titre)	10 ± 33·5	10 ± 59·7	0·381
EA IgM (titre)	0 ± 2·2	0 ± 4·1	0·7
EBNA (titre)	10 ± 69·9	20 ± 63·4	0·678
BZLF1 mRNA in skin lesion, yes/no	1/21	4/2	0·003
BARTs mRNA in PBMCs, yes/no	10/1	3/1	0·476
HSCT, yes/no	3/19	3/4	0·13
EBER *in situ* score (mean)	1·44	2	0·20
CD56+ infiltrating cells, yes/no	5/11	1/3	1·00
γδT cells in PBMCs (%), mean ± SD	13·2 ± 7·8	17·3	0·75
Natural killer cells in PBMCs (%), mean ± SD	30·2 ± 25·2	27·2 ± 22·4	0·86

Values are n unless stated otherwise. Totals vary due to available data. Univariate analysis of factors related to the mortality of classical HV (cHV), systemic HV (sHV), HMB only and HMB + HV. VCA, viral capsid antigen; EA, early antigen; EBNA, Epstein–Barr virus nuclear antigen; BARTs, BamHI A rightward transcripts; HSCT, haematopoietic stem-cell transplantation; EBER, Epstein–Barr virus-encoded small nuclear RNA; PBMC, peripheral blood mononuclear cell. [a]P-values were obtained by use of either Fisher's exact test or the Mann–Whitney U-test. Laboratory data were determined at the time of diagnosis.

The mortality rate for patients under 9 years was 8%, much lower than for those over 9 years, in whom it jumped to 83% (P = 0·001) (Fig. 2). Among patients with cHV and sHV, a poor prognosis was observed in those over 9 years of age (P = 0·0041). Because a good prognosis is expected with cHV, we recalculated the mortality rates excluding patients with cHV. However, the significance of the age-related difference (those under vs. over 9 years) remained (P = 0·0095). This suggests that an onset age > 9 years is a risk factor.

BZLF1 as a molecular indicator of poor prognosis

The expression of EBV-encoded BZLF1 mRNA, an immediate–early gene product, was detected in the skin lesions of patients in the sHV, HMB-only and HMB + HV groups, while no BZLF1 mRNA expression was observed in any of the 13 patients with cHV (Fig. 3a). The positivity rate of BZLF1 mRNA expression was significantly higher in the three groups with systemic symptoms (33%, P = 0·047). No difference was observed in BZLF1 mRNA expression among the three groups.

Regarding EBV-related molecules, the expression of EBV-encoded BZLF1 in the skin lesions was statistically correlated to a poor prognosis (P = 0·003): four of five (80%) patients who were BZLF1 $mRNA^+$, and two of 23 (9%) patients who were BZLF1 $mRNA^-$ died during follow-up (Fig. 3). Among the 25 patients who could be followed up, Kaplan–Meier analysis confirmed that patients positive for BZLF1 mRNA showed a worse prognosis than those who were negative (P = 0·012). The mortality rate excluding patients with cHV was higher in the BZLF1 $mRNA^+$ group (four of five patients, 80%) than in the BZLF1 $mRNA^-$ group (one of nine patients, 11%), but there was no statistical significance (P = 0·37). The survival rate of BZLF1 $mRNA^+$ patients was 80% in the first 5 years, but decreased to 27% in 10 years. No correlation was found in other EBV-related markers, including anti-EBV antibody titres, EBV DNA load in PBMCs, the number of $EBER^+$ cells, or the subsets of infiltrating cells in the cutaneous lesions. Furthermore, neither an increase in the number of γδT cells nor an increase in NK cells among PBMCs was correlated with mortality (P = 0·75 and P = 0·86, respectively).

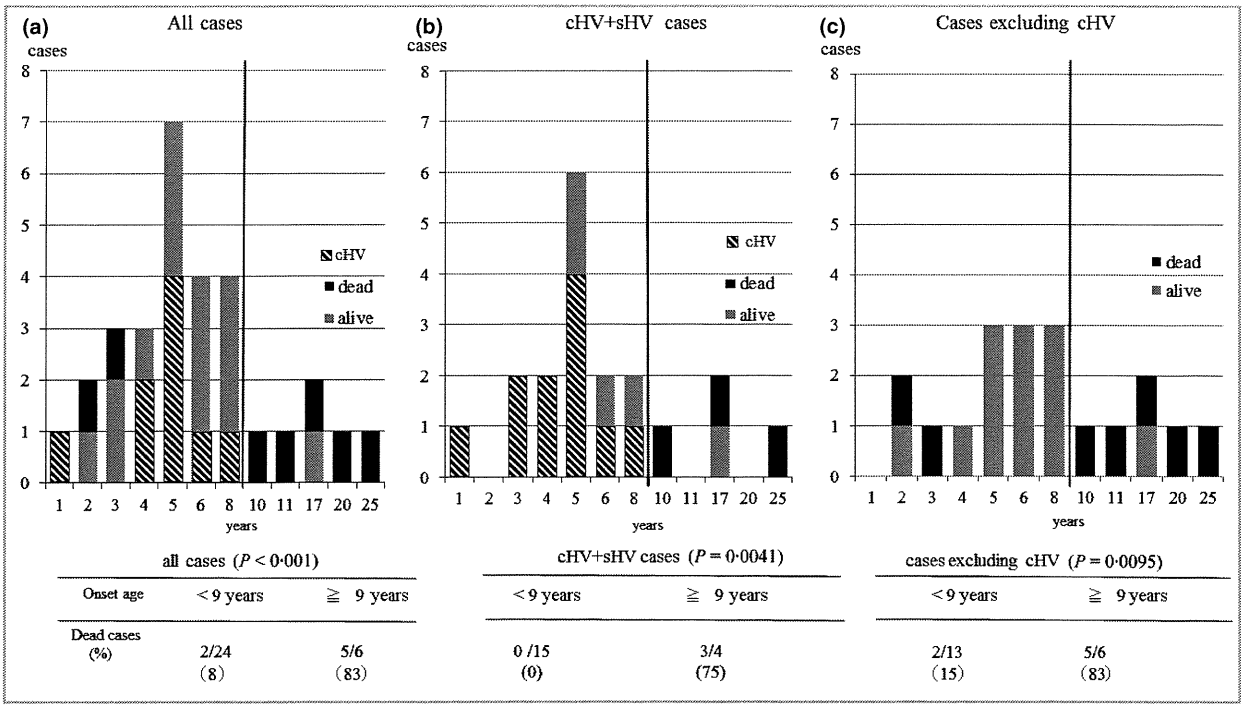

Fig 2. Onset age and mortality rates. (a) There was a clear difference in mortality rates: patients aged under 9 years had mortality of 8%, while those over 9 years had a mortality of 83% (P < 0·001). (b) In the group of patients with classical hydroa vacciniforme (cHV) or systemic HV (sHV), patients aged > 9 years also showed a poor prognosis (P = 0·0041). (c) Even when patients with cHV, which had a favourable outcome, were excluded, the significant difference in mortality rates between patients under and over 9 years remained (P = 0·0095).

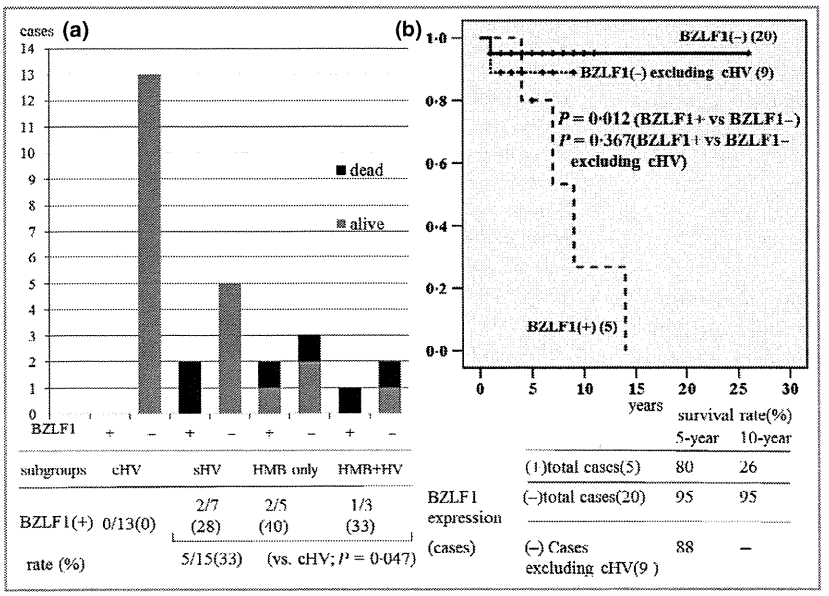

Fig 3. BZLF1 mRNA expression in disease types and prognosis. (a) No BZLF1 mRNA expression was observed in the skin lesions of the 13 patients with classical hydroa vacciniforme (cHV), while BZLF1 mRNA was detected in five of the 15 patients (33%) with sHV, hypersensitivity to mosquito bites (HMB) only or HMB + HV (P = 0·047). The log-rank test demonstrated that BZLF1 mRNA$^+$ cases showed significantly worse prognosis than BZLF1 mRNA$^-$ cases (P = 0·012). (b) Among the 25 patients who were followed up, the 10-year survival rate was significantly worse in the BZLF1 mRNA$^+$ group by log-rank test (27% vs. 95%, P = 0·003).

Discussion

Our research demonstrated that patients with cHV showed a favourable prognosis, with 100% survival, and were distinct in this way from the other three groups (sHV, HMB only and HMB + HV), in which approximately one-third of patients had died by the end of the follow-up period. Therefore, the cHV criteria used for the present study, namely 'typical cutaneous lesions of HV without systemic symptoms or abnormalities in routine laboratory tests results', are valid criteria for distinguishing benign diseases from those with fatal potential. However, it is important to remember that patients with typical HV lesions may progress to having systemic forms with fatal outcomes, as previously reported.[2] In terms of disease progression, HMB only may result in fatal outcome within the first 5 years, sHV may progress later, and cases with episodes of both HV and HMB may also require a longer period for progression.

Our univariate analysis of patients demonstrated two risk factors: (i) age of onset > 9 years ($P < 0{\cdot}001$) and (ii) expression of BZLF1 mRNA in the skin lesions ($P = 0{\cdot}003$). In contrast, no prognostic correlation was observed for EBV-infected lymphocyte subsets, anti-EBV antibody titres, or EBV DNA load, although these parameters would be useful for a diagnostic test or for monitoring of EBV^+ cell numbers.

There was a clear difference in mortality rates between age groups: the rate for patients under 9 years was 8%, while that for patients over 9 years was 83% ($P < 0{\cdot}001$). Because cHV with a favourable prognosis occurred in the first decade, we recalculated the mortality rates excluding patients with cHV. The results still showed a significant difference between the under/over 9 years categories ($P = 0{\cdot}0041$). Therefore, we conclude that onset age may be a risk factor. This supports the findings of a previous report on CAEBV in which onset age > 8 years was one of the prognostic factors.[10] Our analyses indicate that the possibility of disease progression should be considered for patients aged > 9 years at onset.

In addition to late onset, BZLF1 mRNA expression was closely related to more severe disease conditions and poorer prognosis. BZLF1 is an immediate–early gene product that induces EBV reactivation, with subsequent generation of the lytic cycle infection-associated viral antigens, which evoke CTL responses.[16] Although a previous report has described that BZLF1 expression was associated with the proliferation of transformed lymphocytes,[17] our observations indicate that fatal outcomes may be related to haemophagocytic syndrome and multiorgan failure mediated by host immune reactions, but not to the tumour burden evaluated by EBV DNA load or serum lactate dehydrogenase levels. Therefore, BZLF1 may be important both as a pathogenic molecule that accounts for systemic symptoms and as a prognostic marker.

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