

Additional Evidence that Pityriasis Rosea Is Associated with Reactivation of Human Herpesvirus-6 and -7

Francesco Broccolo,* Francesco Drago,† Anna M. Careddu,* Chiara Foglieni,* Laura Turbino,‡ Clementina E. Cocuzza,‡ Carlo Gelmetti,§ Paolo Lusso,*¶ Alfredo E. Rebora,† and Mauro S. Malnati*

*Unit of Human Virology, DIBIT San Raffaele Scientific Institute, Milan, Italy; †Department of Endocrinology and Metabolic Diseases, Section of Dermatology, University of Genoa, Genoa, Italy; ‡Department of Clinical Medicine and Biotechnology, Section of Dermatology, University of Genoa, Genoa, Italy; §Department of Paediatric Dermatology, IRCCS, Ospedale Maggiore, Milan, Italy; ¶Department of Medical Science, University of Cagliari, Cagliari, Italy

To elucidate the role of human herpesvirus (HHV)-6 and -7 (HHV-7) in pityriasis rosea (PR), we measured their DNA load in plasma, peripheral blood mononuclear cells (PBMC), and tissues using a calibrated quantitative real-time PCR assay. We also studied HHV-6- and HHV-7-specific antigens in skin by immunohistochemistry and anti-HHV-7 neutralizing activity using a syncytia-inhibition test. Plasma and PBMC were obtained from 31 PR patients (14 children, 17 adults), 12 patients with other dermatites, and 36 blood donors. Skin biopsies were obtained from 15 adults with PR and 12 with other dermatites. HHV-6 and HHV-7 DNA were detected in 17% and in 39% of PR plasmas, respectively, but in no controls. HHV-7 viremia was associated with a higher PBMC load and, in adults, with systemic symptoms. HHV-7, but not HHV-6, levels in PBMC were higher in PR patients than in controls. HHV-6 and HHV-7 antigens were found only in PR skin (17% and 67% of patients analyzed, respectively), indicating a productive infection. Syncytia-neutralizing antibodies were found in PR patients and controls, but their titers were lower in patients with HHV-7 viremia. These data confirm the causal association between PR and active HHV-7 or, to a lesser extent, HHV-6 infection.

Key words: herpesviruses/immunohistochemistry/pathogenesis/pityriasis rosea/real-time PCR analysis
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Pityriasis rosea (PR) is a common, acute, self-limiting papulosquamous skin disorder. Some of its clinical and epidemiological features suggest the pathogenic role of an infectious agent (Allen *et al*, 1995). In 1997, a link between PR and the reactivation of human herpesvirus (HHV)-7 infection, a ubiquitous β -herpesvirus, was first suggested by two of us (F. D. and A. R.) (Drago *et al*, 1997a, b). Contrasting results were, however, obtained in subsequent studies: several authors were unable to confirm the original findings (Kempf *et al*, 1999; Kosuge *et al*, 2000; Karabulut *et al*, 2002) both in adults and children with PR (Chuh and Peiris, 2001), whereas others suggested that reactivation of HHV-7 and, in some cases HHV-6, may occur in PR (Yasukawa *et al*, 1999; Tanaka-Taya *et al*, 2000). More recently, additional evidence in support of the involvement of HHV-7 and/or HHV-6 in PR came from the detection of herpesvirus-like particles in PR lesional skin (Drago *et al*, 2002), as well as from a study by Watanabe *et al* (2002), who concluded that PR is associated with active systemic co-infection with both HHV-6 and HHV-7.

Unfortunately, most studies used qualitative molecular approaches, such as nested PCR (Drago *et al*, 1997b; Yasukawa *et al*, 1999; Watanabe *et al*, 1999, 2002; Kosuge *et al*, 2000), which, in the case of highly diffused agents like

herpesviruses, cannot provide conclusive evidence for an etiological link. This is particularly true when simple independent epidemiological markers, such as the anti-HHV-6 and anti-HHV-7 IgG antibody titers, are virtually useless given their high seroprevalence in healthy humans (Clark, 2000) and the partial antigenic cross-reactivity existing among herpesviruses (Efstathiou *et al*, 1988).

Stronger evidence is provided, instead, by bona fide calibrated quantitative real-time PCR (CQ-PCR) approaches that are particularly suited for comparing the pathogen load among different cohorts of individuals and different specimens of the same patient, and are less prone to technical artifacts. In this respect, three of us (F. B., P. L., and M. M.) have developed a sensitive and accurate CQ-PCR approach (Locatelli *et al*, 2000; Broccolo *et al*, 2002, 2003) that has been used, in this study, to assess the HHV-6 and HHV-7 DNA load in peripheral blood mononuclear cells (PBMC), plasma, and skin lesions of different cohorts of individuals. PR was studied in both children and adults. The expression of specific HHV-6 and HHV-7 antigens in skin lesions and the presence of anti-HHV-7 neutralizing antibodies (Nab) in the sera of patients and controls were investigated as complementary independent approaches.

Results

Detection of HHV-6 and HHV-7 DNA in plasma and blood cells HHV-6 and HHV-7 plasma viremia, which is a specific marker of active infection, was detected in a significant

Abbreviations: CQ-PCR, calibrated quantitative real-time PCR; HHV, human herpesvirus; IHC, immunohistochemistry; Mab, monoclonal antibodies; Nab, neutralizing antibodies; PBMC, peripheral blood mononuclear cells; PR, pityriasis rosea

Table I. HHV-6 and HHV-7 plasma viremia and cellular load in PBMC and skin from patients and controls

Subjects	No. of individuals	HHV-6 TaqMan assay			HHV-7 TaqMan assay		
		No. (%) of positive samples	No. of genome equivalents		No. (%) of positive samples	No. of genome equivalents	
			× 10 ⁶ cells or mL	Median		× 10 ⁶ cells or mL	Median
PR							
Plasma ^a	31	5/31 (16)	<10–2.6 × 10 ⁶	50	12/31 (39)	<10–150	40
PBMC	31	26/31 (84)	<10–9 × 10 ⁶	35	28/31 (90)	<10–6200	100
Skin	15	7/12 (58)	<10–370	90	10/12 (83)	<10–4.2 × 10 ³	175
Inflammatory diseases ^b							
Plasma	12	0/12 (0)	<15 ^c		0/12 (0)	<15	
PBMC	12	4/12 (33)	10–25	20	9/12 (75)	10–75	60
Skin ^d	12	1/12 (8)	<15		0/12 (0)	<15	
Blood donors							
Plasma	36	0/36 (0)	<20		0/36 (0)	<20	
PBMC	36	13/36 (36)	10–50	30	31/36 (81)	10–725	60

^aIncluding 14 pediatric and 17 adult patients.

^bIncluding patients with atopic dermatitis, acute eczema, drug eruption or erythroderma, acute and chronic urticaria.

^cFor negative samples the highest cut-off value is reported.

^dAdults only.

HHV, human herpesvirus; PBMC, peripheral blood mononuclear cells; PR, pityriasis rosea.

proportion of PR patients (five of 31 and 12 of 31, respectively), but in none of the healthy blood donors and of the patients with other inflammatory diseases (Table I). Plasma viremia for both HHV-6 and HHV-7 was co-detected in two adult patients with PR. Overall, DNA of one or both viruses was found in the plasma of 15 of 31 (48%) of the patients with PR. The frequency of detection of HHV-6 and HHV-7 DNA (26 of 31 and 28 of 31, respectively) was not significantly different in PBMC from PR patients compared with other inflammatory diseases (four of 12 and nine of 12, respectively) and in blood donors (13 of 36 and 31 of 36, respectively), even though detectable levels of HHV-6 were more often seen among PR patients (Table I).

Again, the frequency of HHV-6 (one of 14 children and four of 17 adults; $p = 0.38$) and HHV-7 DNA (six of 14 children and six of 17 adults; $p = 1$) detection in plasma and PBMC (11 of 14 children and 15 of 17 adults for HHV-6, $p = 1$; 13 of 14 children and 13 of 17 adults for HHV-7, $p = 0.79$) was similar in both pediatric and adult patients with PR.

Detection of HHV-7 and HHV-6 in skin samples Skin specimens were positive for HHV-7 DNA in a significant proportion of PR patients (10 of 12 vs zero of 12; $p = 0.01$), but in none of the patients with other inflammatory diseases (Table I). By contrast, the presence of HHV-6 DNA was not significantly higher in the skin of PR compared with other inflammatory diseases (seven of 12 vs one of 12; $p = 0.21$) (Table I). Overall, DNA of one or both viruses was found in 11 of 12 (92%) of the skin biopsies tested, with a frequent co-detection of both viruses (six of 12%, 50%).

Expression of the HHV-7 pp85 antigen was detected by immunohistochemistry (IHC) in eight of 12 (67%) skin samples, indicating the presence of a productive infection

(Fig 1b–f). In contrast, HHV-6 p41 antigen expression was documented in only two of 12 (17%) of the PR skin biopsy specimens (Table II). Overall, HHV-6 or HHV-7 antigens were expressed in nine of 12 (75%) of the PR skin biopsy samples. HHV-7 and HHV-6 antigen-positive cells appeared to be located mainly in the perifollicular and papillary dermis (Fig 1e, f), with the exception of one patient in whom HHV-7 pp85 was also found in rare epidermal cells (Fig 1b). None of the control skin specimens, obtained from 12 patients affected by other cutaneous diseases, was positive for HHV-6 and only one expressed HHV-7 antigens. All the positivities observed by IHC were confirmed by detecting the appropriate viral DNA in the tissue (Table II). The frequency of HHV-6 and HHV-7 DNA detection by PCR was higher than by IHC (seven of 12 vs two of 12; $p = 0.41$ and 10 of 12 vs eight of 12; $p = 0.75$, respectively), but all PCR-negative samples were also IHC negative. HHV-6 pp41-expressing cells were observed together with a few HHV-7 pp85 antigen-positive cells only in one patient who was PCR positive for both viruses in the tissue but not in the plasma.

Viral load of HHV-6 and HHV-7 in clinical samples A quantitative calibrated real-time PCR assay was used for the accurate quantification of HHV-6 and HHV-7 viral load in clinical specimens. Cellular DNA contamination was low in all plasma samples (median, 200 cellular genome equivalent per mL), thus ruling out the possibility that plasma viremia was because of the destruction of latently infected cells during sample manipulation. DNA from PBMC and tissues samples was considered valuable if the reference CCR5 copy number in each PCR reaction was higher than 10⁵ (corresponding to 5 × 10⁴ cells), whereas DNA from plasma

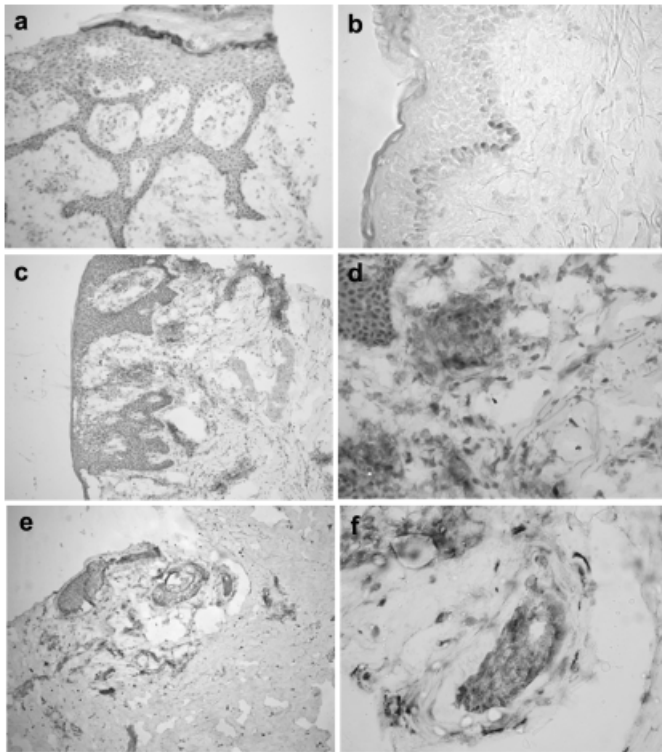


Figure 1
Detection and localization of human herpesvirus (HHV)-7 pp85 protein in skin lesions of four different patients. (a) Patient negative for pp85. (b) Patient displaying positive cells in the epithelial layer. (c) Patient showing absence of positive cells in the epithelium and the presence of positive cells in the subcutaneous derma. (d) Higher magnification of (c). (e) A fourth patient characterized by the presence of positive cells in a perifollicular area. (f) Higher magnification of (e).

samples was considered valuable if the calibrator recovery percentage was higher than 30%. The analysis of the amplification kinetics of the calibrator DNA allowed the presence of PCR inhibitors to be excluded (data not shown) and to determine, for each negative sample, a specific cut-off value for viral DNA detection, which varied between 10 and 35 genome equivalents per mL (Table II).

The level of plasma viremia ranged between 10 and 150 genome equivalents per mL for HHV-7 and between 10 and 2.6×10^6 for HHV-6 (Table I); however, a high viral load for HHV-6 was only observed in a single adult patient (#17), who had 2.6×10^6 and 4200 viral genome equivalents per mL on two subsequent samples (Table II). No significant differences were observed between adults and children with PR (data not shown) as well as among patients infected with a single virus or co-infected (patients #7 and #13, Table II).

Among PR patients, the PBMC viral load of HHV-7 was significantly higher than the HHV-6 load (mean, 917 vs 137 genome equivalents per 10^6 cells; $p=0.037$). The HHV-7 DNA load differed significantly between PR patients and patients with other inflammatory diseases or healthy blood donors, whereas the HHV-6 load in PBMC did not (Fig 2). Remarkably, HHV-7 DNA load was significantly increased in those patients who showed detectable HHV-7 plasma viremia (mean, 2500 vs 535 genome equivalents per 10^6 cells; $p=0.003$). Likewise, the skin HHV-7 DNA load was higher than the HHV-6 one (mean, 741 vs 116 genome

equivalents per 10^6 cells), especially in patients with measurable HHV-7 viremia (mean, 1100 vs 357 genome equivalents per mL). Interestingly, only the HHV-7 load was measurable in the tissues of patients who had detectable plasma viremia for both viruses.

Correlation between anti-HHV-7 serum neutralizing activity, HHV-7 plasma load, and clinical manifestations Bona fide syncytia formation was demonstrated using the CD4⁺ T cell line Sup T1 as a target following infection with an HHV-7 viral stock containing 10^6 cell culture infectious doses (CCID₅₀) per mL. All the human sera screened were found to contain syncytia-neutralizing activity at variable titers. A Gaussian distribution of HHV-7 Nab was observed in both PR patients and in controls, peaking at a titer of 320. Overall, the titers in control plasma were comparable with those of PR patients, irrespective of the clinical phase of the disease (Table III). Interestingly, when PR patients were classified according to their HHV-7 plasma load status, the mean Nab titers was significantly lower in HHV-7 plasma load-positive patients than in HHV-7 plasma load-negative patients (mean = 120 and 456, respectively; $p=0.022$) or in controls (mean, 305; $p<0.001$). In addition, in adult PR patients, HHV-7 and HHV-6 plasma viremia were associated with the presence of systemic symptoms (10 of 20 vs zero of 13; $p=0.020$), but not with the clinical phase of the eruption. In fact, HHV-7 and/or HHV-6 DNA were detected in 10 PR plasma samples, six of which were taken at the first visit and four at the second one.

Discussion

Studies undertaken to assess the etiological role of HHV-7 and HHV-6 in human diseases, such as PR, have been seriously limited by the high prevalence of HHV-6 and HHV-7 infection in the human population (Clark, 2000). Although several diagnostic methods have been developed, they often cannot distinguish between latent (clinically silent) and active (clinically manifest) infection, which is critical to establish a causal relationship between these pathogens and human diseases. Only bona fide quantitative methods, which can measure the HHV-6 and HHV-7 viral load in tissues, blood cells and, particularly, plasma and serum, permit to assess the status of the infection. Three of us have recently developed a fully controlled real-time PCR assay for the measurement of cell-free pathogen DNA load in body fluids, based on the addition of a synthetic DNA calibrator molecule prior to DNA extraction (Locatelli *et al*, 2000; Broccolo *et al*, 2002, 2003). The calibrator permits each sample to be controlled for the presence of PCR inhibitors, allows the determination of cut-off values of sensitivity for negative samples, and normalizes the positive samples for the efficiency of DNA recovery. A similar approach, a TaqMan CQ-PCR assay, has been specifically developed and used in this paper for the detection and quantification of HHV-7 cell-free viremia and cellular viral load.

Cell-free HHV-7 and HHV-6 DNA was detected in plasma of 39% and 16% of patients, whereas none of the 36 healthy blood donors and 12 patients with other inflamma-

Table II Clinical manifestation and virological parameters in adult PR patients

Patient no.	Timing (wk)	Phase	No. of lesions	Relapse	Systemic symptoms ^a	Viremia No. of genome equivalents per mL		Lesional skin			
								No. of genome equivalents per 10 ⁶ cells		Viral antigens	
						HHV-6	HHV-7	HHV-6	HHV-7	HHV-6	HHV-7
1	1	EA	15	No	Yes	<13	<13	129	100	–	+
	3	M	40		Yes	<10	<10				
2	3	M	<15	No	Yes	<10	<10	N/D	N/D	N/D	N/D
	11	PA	0		Yes	<16	<16				
3	2	M	2	No	No	<19	<19	20	820	–	+
	4	PA	4		No	<27	<27				
4	4	PA	30	No	No	<15	<15	N/D	N/D	N/D	N/D
	9	PA	0		No	<20	<20				
5	1	EA	50	No	Yes	<10	<10	370	128	+	+
	7	PA	50		Yes	<17	40				
6	3	M	<10	No	Yes	<22	20	<10	60	–	+
	10	PA	0		No	<25	<25				
7	4	PA	25	Yes	Yes	<19	30	<10		–	+
	11	PA	0		Yes	50	40		1100		
8	2	M	30	Yes	Yes	<10	<10	90	<10	–	–
	6	PA	0		No	<20	<20				
9	2	M	25	No	No	<12	<12	<10	<10	–	–
	6	PA	0		No	<20	<20				
10	6	PA	<20	No	No	<14	<14	<10	50	–	+
	12	PA	0		No	<10	<10				
11	1	EA	12	Yes	Yes	<35	60	150	80	–	+
	6	PA	20		Yes	<12	<12				
12	6	PA	>40	Yes	Yes	<13	25	10	4200	–	+
	12	PA	0		Yes	<16	<16				
13	2	M	<10	No	Yes	<12	<12	<10	200	–	–
	12	PA	>100		Yes	25	20				
14	4	PA	0	Yes	No	<23	<23	N/D	N/D	N/D	N/D
	10	PA	<10		Yes	<12	<12				
15	3	M	>100	Yes	Yes	20	<10	40	15	+	–
	15	PA	>100		Yes	N/A	N/A				
16	1	EA	<15	No	No	<10	<10	N/A	N/A	N/A	N/A
	9	PA	0		No	<12	<12				
17	2	M	>100	No	Yes	2.6 × 10 ⁶	<13	N/A	N/A	N/A	N/A
	12	PA	<15		Yes	4200	<10				

^aSystemic symptoms included: fatigue (in all patients), headache (in patients #1, 2, 7, 8, 11, 13, 15, 17), insomnia (in patients #2, 8, 15), arthromyalgia (in patients #1, 5, 15), angina (in patients #5), irritability (in patients #13).

EA, early acute; PA, post-acute; M, manifest; N/A, not available; ND, not determined; HHV, human herpesvirus; PR, pityriasis rosea.

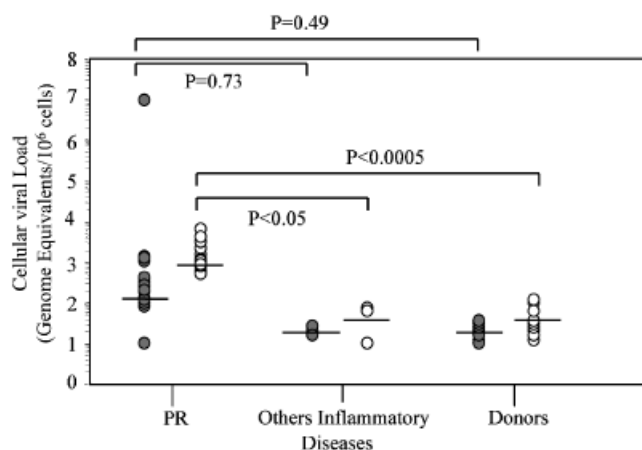


Figure 2
Peripheral blood mononuclear cells (PBMC) viral load of human herpesvirus (HHV)-6 (closed circles) and HHV-7 (open circles) expressed as genome equivalents for 10^6 of cells. The bars indicate the mean DNA load copy numbers in each group (pityriasis rosea (PR), other inflammatory diseases, and blood donors) of TaqMan-positive samples. The HHV-7 load in PBMC differed significantly among PR patients, other inflammatory diseases, and blood donors ($p < 0.05$); by contrast, the HHV-6 load in PBMC was not significantly different in any of the groups considered.

tory skin diseases were positive. Our results confirm that both HHV-7 and HHV-6 do not commonly replicate in healthy individuals (Secchiero *et al*, 1995) as well as in patients affected by several acute or chronic cutaneous inflammatory disorders. But we documented the active replication of these herpesviruses in PR patients regardless of their age. Indeed, plasma viremia was detected in adults and children with the same frequency for both viruses. We detected HHV-7 and HHV-6 DNA in PR plasma less frequently than Watanabe *et al* (2002), who observed cell-free viral DNA in 100% and 88% of serum samples, respectively. This discrepancy could be related to the combination of two variables, namely the type of biological material studied and the different methods of investigation used. In short, Watanabe *et al* (2002) evaluated only serum samples positive for the amplification of the human β -globin gene. This implies the presence, in the sample, of a significant amount of human genomic DNA likely because of the destruction of leukocytes that occurred during the sample manipulation or to a contamination with white blood cells. In addition, qualitative approaches, such as highly sensitive nested PCR techniques, cannot evaluate the weight of viral

DNA contaminations that originated from latently infected cells. On the contrary, we used fully controlled quantitative assays to measure, at the same time, viral load in both PBMC and plasma, controlling to ensure that plasma samples were almost devoid of human DNA as well. Under these conditions, the contribution to HHV-6 and HHV-7 detection by viral DNA sequences belonging to latently infected PBMC is meaningless. Therefore, only plasma DNA load measurements can be considered a bona fide direct marker of viral replication. Overall, the levels of HHV-6 and HHV-7 plasma load proved to be similar and particularly low. This fact makes detection of these viruses cumbersome and may help to explain why the data of literature are discordant.

The significant Nab response against HHV-7 indicates that, in PR patients, HHV-7 replication is associated with an endogenous reactivation/reinfection, but not with a primary HHV-7 infection, even in children. In this respect, our data are in agreement with Watanabe *et al*'s (2002) observation, who, finding HHV-7 and HHV-6 DNA in the saliva of all their patients, excluded a primary HHV-6/HHV-7 infection, since salivary gland cells act as a reservoir for HHV-7 and/or HHV-6 only in previously infected individuals (Sada *et al*, 1996). Moreover, finding a neutralizing humoral response suggests that, in PR, immune cell-mediated responses are probably crucial in affecting HHV-7 reactivation, as it is the case in other viral diseases. Despite this, the inverse correlation between titers of anti-HHV-7 neutralizing activity and detection of HHV-7 in PR plasma may imply that humoral responses contribute to limit viruses to spread, lowering their level in the blood and controlling the extent of the disease. The observation that HHV-7 plasma viremia is strictly associated with the severity of the symptoms strengthens such a hypothesis.

Remarkably, in all adult patients in whom plasma viremia was detected, viral DNA and, in many instances, also viral-encoded proteins belonging to HHV-6 or HHV-7 were consistently found also in skin lesions. PCR and IHC yielded slightly discordant results, however, probably because PCR measures viral DNA regardless of the viral life cycle, whereas IHC detects viral antigens express only during a given moment of the virus replication cycle.

The expression in the skin lesions of the HHV-7 phosphoprotein complex pp85, a structural protein involved in the late stage of infection (Kempf *et al*, 1998), and that of the HHV-6 p41 nuclear phosphoprotein (Chang and Balachandran, 1991) indicates that, in agreement with Watanabe *et al*'s (2002) *in situ* hybridization data, a productive viral infection is occurring. The significant difference found in the number

Table III. Titration of syncytia-inhibitory antibodies to HHV-7 in human sera

PR subjects	No. of samples tested	Titers of syncytia-inhibitory antibodies in serum						
		20	40	80	160	320	640	1280
Children	14			2 ^a	3	6	3	
Adults	17		1	2	4	6	3	1
Other inflammatory diseases	12				2	6	4	
Healthy adults	36	1	4	5	7	11	7	1

^aNumber of patients with the indicated syncytia-inhibitory titer.
HHV, human herpesvirus; PR, pityriasis rosea.

of pp85- and pp41-expressing skin biopsies (67% and 17%, respectively) strongly suggests that, in PR, productive HHV-7 infection is more frequent than HHV-6 infection. This finding is further confirmed by the higher percentage of HHV-7 viremic patients as well as by the significant difference in PBMC load between the two viruses.

In contrast to Watanabe *et al* (2002), in our study, HHV-6 and HHV-7 dual infection occurred only in 6% of plasma samples analyzed, although DNA sequences belonging to both viruses were present in a significant fraction of the tissue samples. We conclude, therefore, that, although possible, the HHV-7/HHV-6 interaction may not represent a common pathogenic mechanism in PR patients. Interestingly, in our dually infected patients, HHV-7 replication preceded the appearance of HHV-6, as indicated by the presence of the isolated HHV-7 in the skin biopsy and, in one case, also in the plasma sample obtained at the first time-point, suggesting that HHV-7 replication may favor, *in vivo*, HHV-6 reactivation. These data are in agreement with the *in vitro* observation of a frequent reactivation of endogenous HHV-6 in PBMC cultures exposed to HHV-7 *ex vivo* (Katsafanas *et al*, 1996). On the other hand, we documented in three distinct cases the active replication of HHV-6 alone, thus demonstrating that this agent may cause a clinically indistinguishable disease.

In conclusion, we have demonstrated that HHV-7 and, less frequently, HHV-6 are active during the early stage of PR, suggesting that they might play, both in adults and in children, an etiological role in this disease. In addition, we have shown that HHV-6 and HHV-7 plasma load, a direct marker of viral replication, is associated with the development of systemic symptoms as well as with a significant reduction of the humoral neutralizing response against HHV-7, further suggesting that PR may be because of the endogenous reactivation of HHV-7 or HHV-6 infection.

Material and Methods

Patients, samples collection/preparation Thirty-one consecutive patients with PR, 17 adults and 14 children (median age: 39 and 9 y, respectively), were recruited between December 2001 and December 2003 at the Section of Dermatology, University of Genoa and at the Department of Pediatric Dermatology, University of Milan. All patients had the classical clinical findings of PR and were otherwise healthy and immunocompetent. Among adults, 12 complained of systemic symptoms during the course of PR. Systemic symptoms had different degrees of severity and duration. In some patients, severe symptoms were present from the prodromal phase up to the resolution of the skin eruption and even beyond. Fatigue was present in all patients, but other symptoms, such as headache, insomnia, arthralgias, myalgias, pharyngitis, and irritability, were common as well.

The clinical status of the patients was defined as early acute phase (within 1 wk from eruption), manifest phase (7–21 d from eruption), and post-acute or convalescent phase (21 d from eruption). This permitted the association between plasma viremia, the phase of the disease, and clinical manifestations (presence or absence of systemic symptoms, number of lesions, etc.) to be studied.

A total of 212 blood and tissue samples were tested assessing both HHV-6 and HHV-7 DNA load. Blood samples were taken from all 17 adult patients during the first visit and after a few weeks of follow-up (median = 5, range = 1–15). At the first visit, most adult patients consented to a punch-biopsy of lesional skin (15 of 17). The skin biopsies were taken from secondary lesions, except in

one patient in whom the “herald” patch was taken. Biopsy samples were included in TissueTek O.C.T. (Bayer Corporation, Pittsburgh, Pennsylvania) and snap-frozen in iso-pentane/liquid nitrogen. Specimens were immediately stored at -80°C . PBMC, plasma, and sera were also obtained from 14 children during the early/manifest phase of the disease. Blood and tissue samples from 12 patients with other inflammatory disorders (atopic dermatitis, acute eczema, drug eruptions, acute and chronic urticaria, psoriasis) were studied as controls. In addition, blood samples were obtained from 36 healthy blood donors.

The medical ethical committee of the Universities of Genoa and Milan approved all the described studies. The study was conducted according to the Declaration of Helsinki principles. All patients or the patients' parents gave their written informed consent.

PBMC were isolated by density gradient centrifugation of EDTA-treated whole blood. DNA was extracted from plasma and PBMC by the phenol-chloroform protocol as previously described (Locatelli *et al*, 2000). Plasma samples were subjected to high-speed centrifugation ($26,000 \times g$, 60 min, 4°C) to concentrate virions. Five to ten biopsy sections (10 μm thick), each parted from the following by 100 μm , were pooled and DNA was extracted after proteinase K digestion (10 mg per mL) in 400 μL of Tissue Buffer (50 mM Tris-HCl, pH 8.0; 200 mM NaCl; 20 mM EDTA, pH 8.0; 1% SDS) and incubated overnight at 56°C .

Calibrated quantitative real-time PCR assays for HHV-6, HHV-7 CQ-PCR-based assays were performed on an ABI PRISM 7700 SDS (Applied Biosystems, Foster City, California), as reported elsewhere (Broccolo *et al*, 2002). Four independent TaqMan real-time PCR assays were performed to detect HHV-6, HHV-7, calibrator, and a highly conserved fragment of the single-copy human CCR5 gene; this quantitative detection system was used to quantify the content of human genomic DNA both in plasma and in the PBMC pellets (Broccolo *et al*, 2002; Biswas *et al*, 2003).

At least 1 μg of genomic DNA recovered from each PBMC pellet was subjected to real-time PCR analysis. Serial dilutions of genomic DNA purified from PBMC of blood donors were used to generate a reference curve for CCR5 quantification.

Primers and probe for HHV-6 DNA load measurement were previously described (Locatelli *et al*, 2000).

Primers TAQ 7C (5'-AGCGGTACCTGTAAAATCATCCA-3') and TAQ 7D (5'-AACAGAAACGCCACCTCGAT-3') were selected using Primer Express software (PE Biosystem, Foster City, California). They amplify a 129 bp fragment of the minor capsid protein of HHV-7 (from nucleotides 100483 to 100611; GeneBank accession #AF 037218). A fluorogenic probe of 30 bp (5'-ACCAGTGAGAA-CATCGCTCTAACTGGATCA-3'), complementary to an internal region 32 bp downstream of the forward primer, was designed as well. An extensive search of several databases, including the EMBL and GeneBank databases, indicated that neither the primers nor the probe shared significant homology with other known nucleotide sequences. The results were normalized using a synthetic DNA calibrator molecule (10^4 copies per reaction), added to the samples before the extraction step, to control the inter-sample extraction efficiency and to monitor PCR artifacts. The calibrator molecule was then co-amplified by real-time PCR with the HHV-6 and HHV-7 target sequences. The primers and the calibrator probe were specifically selected to avoid cross-hybridization with HHV-6 and HHV-7 systems. All reactions were optimized to obtain the best amplification kinetics under the same cycling conditions (2 min at 50°C , 15 min at 95°C , and 40 cycles of 15 s at 95°C and 1 min per cycle at 60°C) and composition of the reaction mixture as previously described (Broccolo *et al*, 2002). Each sample was tested in triplicate and the mean value was calculated.

IHC assays for HHV-6 and HHV-7 Three to ten serial cryosections (10 μm thick) were stained using hematoxylin and eosin (HE) (Bio-Optica, Milano, Italy) for histological evaluation and, subsequently, IHC for localization of HHV-6 or HHV-7 antigens. Three biopsy samples (patients 2, 4, 14, Table II) were eliminated during a pre-

liminary histological evaluation because they did not contain valuable material (complete absence of the epidermis).

Monoclonal antibodies (Mab) 9A5D12 (Chang and Balachandran, 1991) against the HHV-6 p41 early/late protein, expressed in the host cell nucleus and 5E1 (Foà Tomasi *et al*, 1996; Stefan *et al*, 1997) against the HHV-7 pp85 protein, a cytoplasm-expressed antigen, were applied. Briefly, in IHC endogenous peroxidase was inhibited by applying 0.3% H₂O₂. Sections' pre-incubation with a suitable pre-immune serum blocked unspecific binding. Afterwards, the primary antibodies were incubated overnight at 4°C and revealed with a biotinylated antibody followed by avidin-biotin-HRP complexes (ABC elite system, Vector, Vector Labs, Burlingame, California) and diaminobenzidine chromogen-developing solution. For each patient, at least three HE and five to six IHC sections were independently evaluated by two pathologists at light microscope (BH2, Olympus, Melville, New York) and digital pictures were taken with an Olympus C4040 zoom digital camera (Olympus Europe, Planegg, Germany).

Syncytia-inhibition test To identify anti-HHV-7 Nab, a syncytia-inhibition test was carried out, as previously described (Secchiero *et al*, 1994), in all sera samples included in this study.

Briefly, 2×10^5 HHV-7-infected Sup-T1 cells were incubated with serial 2-fold dilutions of the tested sera and then co-cultured with 2×10^5 uninfected Sup-T1 cells in 24-well plates (Costar Corning Costar, Miescourt, Switzerland) in a total volume of 1 mL. The Nab titer was determined as the reciprocal of the highest dilution of sera that completely prevented giant cell formation. Syncytia formation was scored at 20 and 40 h after co-cultivation.

Statistical analysis Fisher's exact test was used to analyze the significance of the different HHV-7 and HHV-6 prevalent in PR patients and controls, and the significance of the different prevalence of clinical manifestations in viremic patients and non-viremic patients. A two-tailed Student's *t*-test was used to evaluate the significance of the different viral loads in the various groups of individuals considered and the different titers of HHV-7 Nab present in PR patients and controls. A $p < 0.05$ value was considered to be statistically significant.

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Address correspondence to: Alfredo E. Rebora, Clinica Dermatologica, Viale Benedetto XV, 7, 16132 Genova, Italy. Email: rebdermo@unige.it

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