Diacron-Reactive Oxygen Metabolites Levels Are Initially Elevated in Patients with Bullous Pemphigoid

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ROS are involved in the pathogenesis of bullous pemphigoid (BP), but this involvement has not been fully elucidated. In this study, to further elucidate the pathogenic role of ROS in BP, we examined the results of the diacron-reactive oxygen metabolite test and the biological antioxidant potential test for 16 patients with BP who visited our hospital before being treated with systemic corticosteroids. In the patients with BP, the average diacron-reactive oxygen metabolite levels, expressed in Carratelli units, were significantly reduced at 1 month of treatment (from 335.6 ± 40.3 Carratelli units to 224.7 ± 61.6 Carratelli units, $P < .001$). Bullous Pemphigoid Disease Area Index (erosions/blisters) scores correlated with diacron-reactive oxygen metabolite levels ($r = 0.51$), suggesting that those levels reflect the disease severity. We also performed staining of 3,5-dibromotyrosine in skin tissues. The 3,5-dibromotyrosine is expected to be a marker of tissue damage related to inflammation and allergies. The 3,5-dibromotyrosine was stained in infiltrated cells around the dermis, throughout the blister fluid, and at the basement membrane within the blister. It is considered that tissue destruction caused by the myeloperoxidase released from neutrophils and by eosinophil peroxidase released from eosinophils is involved in blister formation. The results suggest that ROS play a role in BP.

Keywords: Bullous pemphigoid, Diacron-reactive oxygen metabolites, Dibromotyrosine, Oxidative stress, ROS

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INTRODUCTION

Bullous pemphigoid (BP) is an autoimmune bullous disease of the skin characterized by subepidermal blister formation due to autoantibodies to the hemidesmosomal antigens BP180 and BP230 (Schmidt and Zillikens, 2013; Thoma-Uszynski et al, 2004), and it is associated with eosinophilic and neutrophilic infiltration (Jordon et al, 1985; Limberg et al, 2023). Eosinophils and neutrophils release ROS, which are associated with dermal–epidermal separation (Amber et al, 2018; de Graauw et al, 2017; Yu et al, 2010). ROS are a group of highly reactive molecules derived from oxygen, including oxygen-free radicals and nonradical species (Herb et al, 2021). They are oxygen-containing radicals that are capable of independent existence with 1 or more unpaired electrons. Some prominent ROS include superoxide, hydrogen peroxide (H_2O_2), hydroxyl radical, and singlet oxygen (Li et al, 2016; Sies et al, 2022). They function as signals that turn on and off biological functions and are intermediates in reduction–oxidation. Although they are important in many biological processes, they can also cause cellular damage by affecting lipids, DNA, RNA, and proteins. The balance of ROS is crucial for normal physiological function, and an imbalance can lead to oxidative stress and various diseases (Hitomi et al, 2022; Pigazzani et al, 2022; Tozaki et al, 2022).

The derivatives of reactive oxygen metabolites (diacron-reactive oxygen metabolites [d-ROMs]) test is a widely used assay for measuring oxidative stress in biological samples, particularly in serum or plasma. This test does not directly measure reactive oxygen and free radicals but evaluates the oxidative stress by quantifying the metabolite hydroperoxides (Hitomi et al, 2022; Pigazzani et al, 2022). Hydroperoxides are highly stable even in stored samples (Cavalleri et al., 2004; Matsuo et al, 2024). Measurement is based on the reaction of the peroxides in the sample, which oxidize iron in an acid, and the newly formed radicals then oxidize N,N-diethyl-para-phenylendiamine, resulting in a pink color with a maximum absorbance at 505 nm (Hitomi et al, 2022; Li et al, 2016). The unit of measurement for the d-ROMs test is the Carratelli unit (U.CARR), with 1 U.CARR equaling 0.08 mg H_2O_2/dl (Ito et al, 2017). The d-ROMs test reflects oxidative stress, whereas the biological antioxidant potential (BAP) test reflects antioxidant capacity. The BAP test is used to assess the antioxidant capacity of biological samples, such as

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Abbreviations: BAP, biological antioxidant potential; BP, bullous pemphigoid; BPDAI, Bullous Pemphigoid Disease Area Index; DiBrY, 3,5-dibromotyrosine; DIF, direct immunofluorescence; d-ROM, diacron-reactive oxygen metabolite; EPO, eosinophil peroxidase; H_2O_2, hydrogen peroxide; IHC, immunohistochemical; U.CARR, Carratelli unit

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blood or serum. It measures the reducing power from iron (III) to iron (II) in a biological sample, reflecting the sample's ability to eliminate free radicals and counteract oxidative stress (Kasote et al, 2015; Munteanu and Apetrei, 2021). A higher BAP value indicates a greater capacity to neutralize harmful ROS and prevent cellular damage. Although the d-ROM test and the BAP test have been used in many diseases, they have not yet been reported in patients with BP.

The anti−3,5-dibromotyrosine (DiBrY) is a biomarker for tissue damage associated with inflammatory and allergic disorders (Van Dalen et al, 2009; Wu et al, 2000). DiBrY forms as a result of the reaction of hypohalous acid on proteins bearing Y, leading to the formation of 3,5-dibromotyrosine, after which tissue damage occurs (Kato et al, 2005). To investigate the contribution of oxidative stress to BP pathogenesis, we measured the d-ROMs and BAP levels before treatment and at 1 month of treatment with systemic corticosteroids in patients with BP. Next, to visualize ROS, we tried to detect oxidative products using the anti-DiBrY antibody on lesional skin.

RESULTS

In patients with BP, the d-ROM levels increased early in the disease and decreased with treatment

We enrolled 16 patients with BP who had visited our hospital before being treated with systemic corticosteroids between 2017 and 2023. Their serum d-ROM and BAP levels were evaluated before treatment and at 1 month of treatment. The patient information is summarized in Table 1 (7 males, 9 females; mean age = 77 years). The average d-ROM levels were found to be significantly reduced at 1 month of treatment (from 335.6 ± 40.3 U.CARR to 224.7 ± 61.6 U.CARR, $P < .001$, paired *t*-test) (Figure 1a and Table 2). The BAP levels were within normal limits in 11 of the 16 patients and were decreased at 1 month of treatment (from 2102.8 ± 340.9 μmol/l to 1869.8 ± 426.1 μmol/l, $P = .09$, paired *t*-test) (Figure 1b and Table 2).

In patients with BP, the d-ROM levels correlated with Bullous Pemphigoid Disease Area Index but did not correlate with titers of anti-BP180 or anti-BP230 antibodies

Because high levels of ROS in BP may play a role in disease activity, we next examined the d-ROM levels and the disease activity as measured by Bullous Pemphigoid Disease Area Index (BPDAI). We retrospectively scored the BPDAI before treatment and at 1 month of treatment and then determined the absolute rate of change. As shown in Table 2 and Figure 2j and k, a positive moderate correlation was found between the d-ROM levels before treatment and the total BPDAI score ($r = 0.51$), and among the 3 BPDAI subscores, the correlation was the highest for BPDAI (erosions/blisters) ($r = 0.51$). However, there was no correlation between BPDAI (urticaria/erythema) and the d-ROM levels before treatment ($r = 0.14$). In addition, there was a low positive correlation between the initial amount of corticosteroid administered and the d-ROM levels before treatment ($r = 0.39$). Titers for autoantibodies such as anti-BP180 and anti-BP230 did not significantly correlate with the d-ROM levels

Table 1. Summary of the 16 Patients with BP

			Peripheral Blood (Cell/μl)					Eosinophil Count (Cell/ 500 × 400 μm²)			Antibody Titer (U/ml)		
Case	Age, yr	Sex	WBC	Neut	Lymp	Eosi	NLR	Below	Lateral	Within	BP180	BP230	Complications
Pt1	80	F	16,000	N/A	N/A	N/A	N/A	25	8	17	10,000	0.3	
Pt2	87	F	7320	3886	2408	168	1.61	3	0	11	8.1	26	RA
Pt3	82	F	10,060	9154	704	0	13.00	13	27	21	3020	157	
Pt4	77	M	5480	3720	1370	60	2.72	34	17	40	4.2	74.6	
Pt5	69	F	13,780	N/A	N/A	N/A	N/A	14	43	82	259	29	HL
Pt6	51	M	10,980	7720	2042	132	3.78	19	28	8	935	4.5	Psoriasis
Pt7	85	F	10,400	9131	156	936	58.53	37	26	29	38.3	1.8	DM, HTN, HL
Pt8	83	M	6780	4930	1180	217	4.18	18	21	24	0	93	
Pt9	82	F	9800	5047	3136	1421	1.61	85	117	82	10.7	86.8	
Pt10	73	F	8170	6280	1348	57	4.66	22	16	17	50.8	0.1	
Pt11	79	F	9960	7690	1384	408	5.56	23	18	11	0	118.4	
Pt12	71	M	10,120	4530	1301	112	3.48	43	50	41	988	3.9	DM
Pt13	59	M	15,240	10973	2057	1524	5.33	48	46	34	467	66.2	
Pt14	85	F	8480	5003	1018	1993	4.91	8	60	15	558	63.2	Alzheimer
Pt15	84	M	5870	3434	939	822	3.66	44	9	17	51.8	50	DM, HTN
Pt16	80	M	9230	5261	692	2769	7.60	19	20	35	313	81	DM
R	—	—	0.21	0.14	0.21	−0.71	0.18	−0.22	−0.17	−0.02	0.25	−0.14	
Avg	76.7	—	9854.4	6197.1	1409.6	758.5	8.1	26.7	29.7	28.4	447	53.5	
SD	10.0	—	3053.7	2350.7	775.9	866.3	14.6	20.6	28.2	23.1	787	47.4	

Abbreviations: Avg, average; BP, bullous pemphigoid; DM, diabetes mellitus; Eosi, eosinophil; F, female; HL, hyperlipidemia; HTN, hypertension; Lymp, lymphocyte; M, male; Mono, monocyte; N/A, not available; Neut, neutrophil; NLR, neutrophil/lymphocyte ratio; Pt, patient; RA, rheumatoid arthritis; WBC, white blood cell.

This table shows blood test results and Eosi counts on H&E-stained specimens. We counted the number of infiltrating Eosis stained in H&E within a 550 × 400 μm² under ×400 magnification in 3 locations: just below the blister, lateral to the blister, and randomly within the blister. R shows the correlation coefficient (Pearson's) between before-treatment d-ROM levels and each item. Sex was reported based on self-identification. WBC normal range = 3300 −8600/μl.

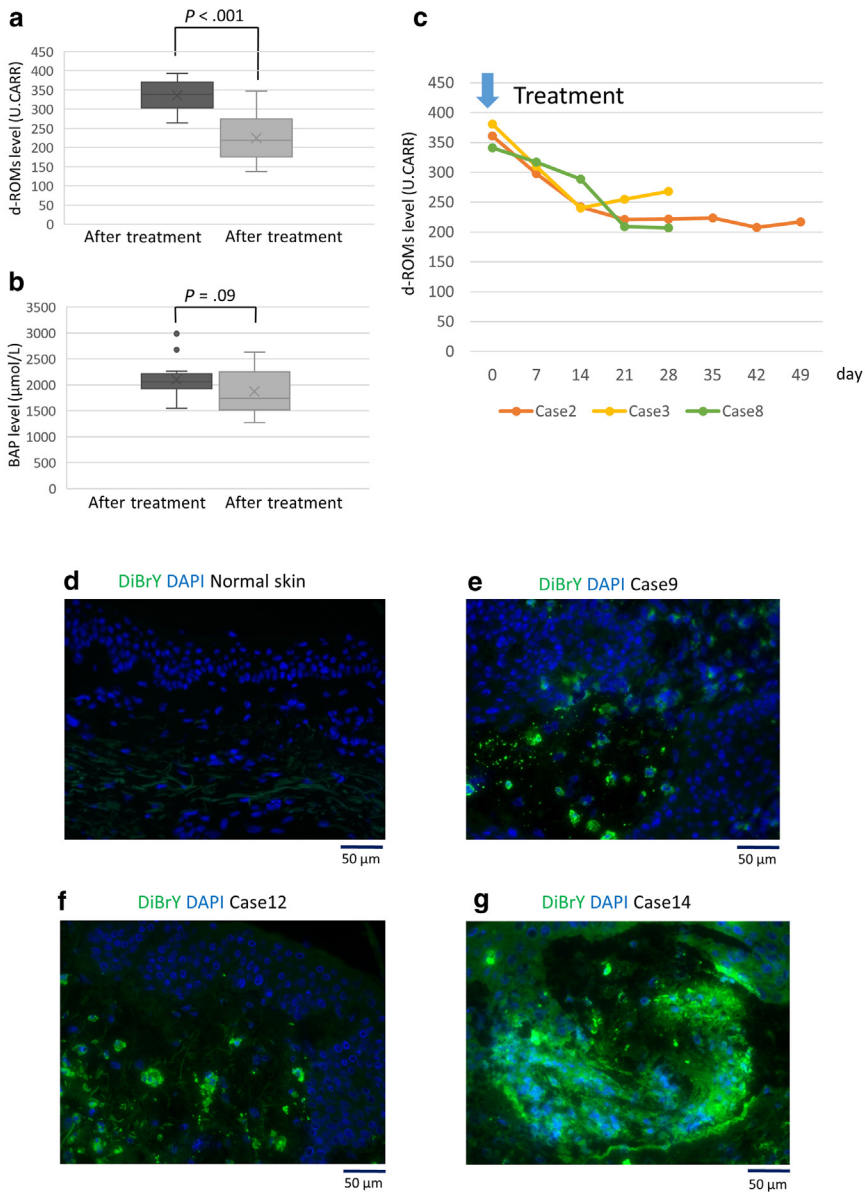


Figure 1. Oxidative stress in patients with pemphigoid. (**a**) Box plot of d-ROMs levels for 16 patients with BP. The d-ROM levels are for before treatment and at 1 month of treatment (from 335.6 ± 40.3 U.CARR to 224.7 ± 61.6 U.CARR, $P < .001$, paired *t*-test). (**b**) Box plot of BAP levels for the 16 patients with BP. The BAP levels are for before treatment and at 1 month of treatment (from 2102.8 ± 340.9 μmol/l to 1869.8 ± 426.1 μmol/l, $P = .0923$, paired *t*-test). The box shows the first and third quartiles, the median (line), and the arithmetic mean (x-mark). The whiskers represent values below the first quartile and above the third quartile within the 1.5-fold interquartile range, respectively. Outliers beyond the whiskers are shown as squares. (**c**) Changes in d-ROM levels in 3 patients with BP at 1 month of treatment with systemic corticosteroids. (**d**–**g**) Direct immunofluorescent staining of DiBrY. Two-color wide-field fluorescence microscopy images of BP erythema for DiBrY (green) and DAPI (blue) are shown. Bar = 50 μm. (**d**) Normal human skin. BAP, biological antioxidant potential; BP, bullous pemphigoid; DiBrY, 3,5-dibromotyrosine; d-ROM, diacron-reactive oxygen metabolite; U.CARR, Carratelli unit.

(Table 1). These results show the d-ROM levels to correlate with clinical severity, particularly for erosions/blisters. Next, we evaluated the d-ROMs levels during the clinical course in 3 patients for whom we had several stored sera. At 1 month of treatment with systemic corticosteroids, the d-ROM levels decreased (Figure 1c). In these 3 patients, the serial measurement of d-ROM levels may reflect the clinical course, at least within each individual.

In patients with BP, the d-ROM levels negatively correlated with peripheral blood eosinophil counts

We speculated that because inflammatory cells could be involved as a source of ROS, eosinophils and neutrophils could be elevated in the peripheral blood. As shown in Table 1, patients with BP had elevated average white blood cell counts (mean = 9854.4/μl; normal = 3300–8600/μl). Unexpectedly, the d-ROM levels correlated negatively with the overall number of eosinophils ($r = -0.71$). In contrast, the d-ROM levels correlated slightly positively with lymphocyte percentage ($r = 0.39$) and neutrophil percentage ($r = 0.33$) (data not shown). The neutrophil-to-lymphocyte ratio did not correlate with the d-ROM levels (Table 1). The peripheral cell counts did not positively correlate with the d-ROM levels. Therefore, we next focused on tissue-infiltrating cells, and we evaluated inflammatory cells, such as eosinophils, in paraffin sections with reference to previous studies (Izumi et al, 2016). No correlation was found between the number of eosinophils in skin tissue and the d-ROM levels (Table 1).

In the skin lesions of patients with BP, DiBrY was stained in infiltrated cells around the dermis, throughout the blister fluid, and at the basement membrane within the blister

At our hospital, when skin biopsies are performed, the blistered area is preserved in paraffin sections, and the periery thematous area is preserved in frozen specimens. In this study, we wanted to observe both the blistered and periery thematous

Table 2. Summary of the 16 Patients with BP

	d-ROMs (U.CARR)		BAP (μmol/l)		BPDAI (Erosion Blister/Erythema/Mucosa/Total)			Treatment	
Case	Before Treatment	1 mo Treatment	Before Treatment	1 mo Treatment	Before Treatment	1 mo Treatment	Absolute Change	Initial Dose (mg)	1 mo Treatment Dose (mg)
Pt1	393	153	1774	1419	45/33/5/85	6/1/0/7	86/96/100/91	PSL60	PSL35
Pt2	381	221	2157	1632	33/11/0/44	4/1/0/5	87/90/—/88	PSL40	PSL40
Pt3	381	275	2003	1712	43/1/0/44	4/0/0/4	90/100/—/90	PSL25	PSL30
Pt4	374	217	2029	1710	24/27/0/51	0/0/0/0	100/100/—/100	PSL45	PSL40
Pt5	361	347	2985	1493	39/33/2/74	2/2/0/4	94/96/100/94	PSL80	PSL55
Pt6	361	271	2266	2482	8/22/0/30	2/2/0/4	100/77/—/83	PSL90	PSL60
Pt7	355	173	1788	1402	10/3/0/13	0/0/0/0	100/100/—/100	PSL50	PSL40
Pt8	341	142	1986	1760	51/17/0/68	0/1/0/1	100/94/—/98	PSL40	PSL35
Pt9	336	291	1910	2634	5/19/0/24	0/0/0/0	100/100/—/100	PSL20	PSL15
Pt10	333	310	2678	2255	33/0/1/34	2/0/0/2	93/—/100/94	PSL50	PSL40
Pt11	320	243	2228	2469	3/10/0/13	0/2/0/2	100/80/—/84	PSL30	PSL30
Pt12	317	137	2102	1273	7/40/0/47	1/6/0/7	85/85/—/85	PSL60	PSL60
Pt13	299	201	2089	1922	20/10/0/30	0/0/0/0	100/100/—/100	PSL20	PSL55
Pt14	292	183	2008	1579	13/26/0/39	0/2/0/2	100/92/—/94	PSL10	PSL30
Pt15	266	201	2094	2222	21/3/0/24	0/3/0/3	100/0/—/87	PSL10	PSL35
Pt16	264	231	1549	1953	7/18/0/25	4/3/0/7	42/83/0/72	PSL60	PSL40
R	—	—	0.18	−0.28	0.51/0.14/—/0.51	—	—	0.39	—
Avg	335.9	224.8	2102	1869.8	22.6/17.0/0.5/40.1	1.4/1.6/0/3.0	92.6/—/—/91.1	43.1	40
SD	40.3	61.6	341.0	426.1	16.0/12.4/1.3/20.6	1.9/1.8/0/2.5	14.3/—/—/7.8	23.5	12.2
t-test	—	$P < .01$	—	—	—	—	—	—	—

Abbreviations: Avg, average; BAP, biological antioxidant potential; BP, bullous pemphigoid; BPDAI, Bullous Pemphigoid Disease Area Index; d-ROM, diacron-reactive oxygen metabolite level; PSL, prednisolone; Pt, patient; U.CARR, Carratelli unit.

The d-ROM and BAP levels, BPDAI scores, and dosages of systemic corticosteroid are shown before treatment and at 1 month of treatment. R shows the correlation coefficient (Pearson's) for before treatment d-ROMs levels and each item. The *t*-test compares the d-ROMs levels before-treatment and at 1 month of treatment, using a paired *t*-test. In cases 14 and 15, the skin symptoms did not improve with the PSL initial dose of 10 mg, and the PSL was increased. Minocycline was used in combination with PSL as the initial treatment in cases 2, 3, 8, 9, 10, and 16. BAP normal is >2000 μmol/l.

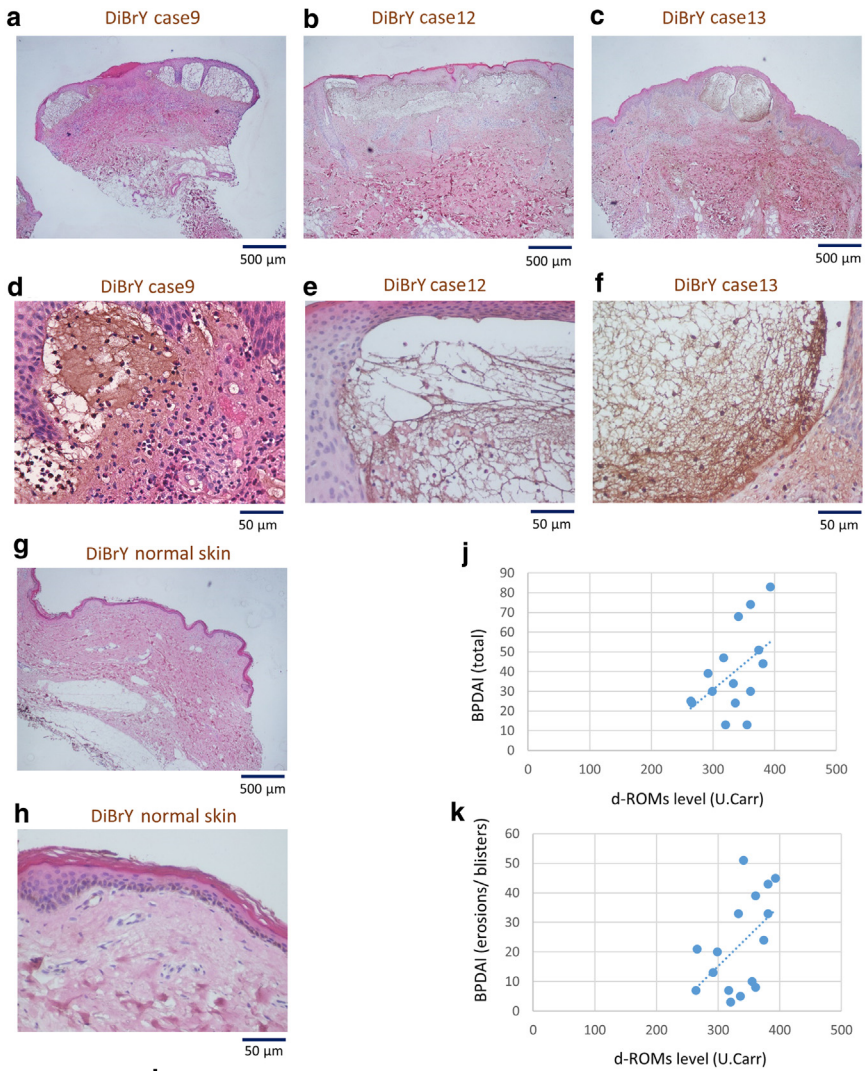
areas, so we performed direct immunofluorescence (DIF) staining using the frozen specimens (Figure 1d–g) and immunohistochemical (IHC) staining using the paraffin sections (Figure 2a–h) for both observations. The results were similar for both stains. In the DIF staining, the DiBrY was stained in infiltrated cells around the dermis, throughout the blister fluid, and at the basement membrane within the blister (Figure 1e–g). In particular, DiBrY-positive cells infiltrating the dermis were well observed (Figure 1e and f). Only case 14 showed blistering with positive DiBrY; there was no blistering in any other case. IHC staining showed DiBrY-positive findings in infiltrated cells around the dermis, throughout the blister fluid, and at the basement membrane within the blister (Figure 2a–f).

The results of DiBrY staining for the 16 patients with BP are statistically presented in Figure 2i. For some of the 16 patients, samples were unavailable because some samples had been used up. Twelve of the 14 (85.7%) tested patients were positive for DiBrY-infiltrated cells around the dermis by DIF, 14 of the 16 (87.5%) tested patients were positive for DiBrY throughout the blister fluid by IHC analysis, and 14 of the 16 (87.5%) tested patients were positive for DiBrY at the basement membrane within the blister by IHC analysis (Figure 2i).

DISCUSSION

Our initial speculation was that inflammatory cells could be involved in BP as a source of ROS, which would be observable as elevated eosinophils and neutrophils in peripheral blood. Contrary to our expectations, the d-ROM levels and peripheral blood eosinophil counts correlated negatively. We considered 2 reasons for this. First, the d-ROM levels are measured for serum and do not reflect the condition of localized tissue, such as blisters. Second, in other diseases, a negative correlation has been observed between activity and the main blood cells involved in disease in the peripheral blood. For example, several studies have indicated that decreased numbers and increased activity of peripheral basophils correlate with disease activity in patients with systemic lupus erythematosus (Liang et al, 2015; Pellefigues et al, 2018). These studies indicated that basophils and their activation status may be associated with disease activity in systemic lupus erythematosus, highlighting the potential significance of basophils in the pathogenesis of the disease and their utility as a biomarker for severe manifestations such as lupus nephritis. It is considered that the main blood cells involved in the disease migrate to local areas and decrease in the peripheral blood.

In our results, the d-ROM levels correlated moderately with the BPDAI scores for erosions/blisters and with total BPDAI. At 1 month of treatment with systemic corticosteroids, the d-ROM levels were decreased. In these 3 patients, the serial measurement of d-ROM levels may reflect the clinical course, at least within each individual. The measurement of d-ROMs is more useful within patients than between patients in tracking clinical progress.



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	Positive for DiBY
Infiltrated cells around the dermis (DIF)	12/14(85.7%)
Throughout the blister fluid (IHC)	14/16(87.5%)
Basement membrane within the blister (IHC)	14/16(87.5%)

Figure 2. Oxidative stress in patients with pemphigoid. (**a**–**f**) IHC staining of DiBrY. The upper row is a low magnification of the blister formation area (×40). Bar = 500 μm. The lower row is a high magnification of the blister area (×400). Bar = 50 μm. (**g**) Normal human skin with DiBrY staining (×40). Bar = 500 μm. (**h**) Normal human skin with DiBrY staining (×400). Bar = 50 μm. (**i**) Positive rate of DiBrY in immunostaining (DIF and IHC). (**j**) Scatterplot of BPDAI scores (total) and d-ROM levels. (**k**) Scatterplot of BPDAI scores (erosions/blisters) and d-ROM levels. Scatterplot showing a moderate positive correlation (r = 0.51; Pearson's correlation coefficient). The dotted line is the trend line. BPDAI, Bullous Pemphigoid Disease Area Index; DiBrY, 3,5-dibromotyrosine; DIF, direct immunofluorescence; d-ROM, diacron-reactive oxygen metabolite; IHC, immunohistochemistry.

In a previous paper, myeloperoxidase from neutrophils prefer using chloride to produce hypochlorous acid, whereas eosinophil peroxidase (EPO) from eosinophils prefer using bromide to produce hypohalous acid (O'Connell et al, 2011). The EPO–H_2O_2–bromide system refers to the EPO-catalyzed reaction that utilizes H_2O_2 and bromide to generate hypohalous acid and other brominating oxidants (Van Dalen et al, 2009; Wu et al, 2000, 1999). This system has been implicated in the pathogenesis of various conditions, including asthma, allergic inflammatory disorders, and helminthic infections. The EPO–H_2O_2–bromide system is involved in the bromination of protein Y residues, leading to the formation of 3-bromotyrosine and 3,5-dibromotyrosine, which are considered major products of protein oxidation by this system. These brominated Y residues can serve as markers for eosinophil-dependent tissue injury in vivo (Van Dalen et al, 2009; Wu et al, 2000). It could be interpreted that EPO released from eosinophils and myeloperoxidase released from neutrophils induce protein Y halogenation, resulting in DiBrY production that leads to tissue destruction and blister formation in the skin of patients with BP.

With regard to pemphigoid, because anti-BP180 and anti-BP230 antibodies are biomarkers of activity, measurement of d-ROMs may have little clinical significance, but it may have research significance. However, if future research reveals more about the relationship between oxidative stress and pemphigoid, its significance will change. Because there are only 16 cases in this study, it is difficult to draw conclusions, and more research is needed. Potential limitations include the retrospective nature of the data, the single-center nature, and the small sample size. In addition, the d-ROM levels are already known to increase as a result of various factors, such as aging, diabetes, cardiovascular disease, and cancer (Hitomi et al, 2022; Pigazzani et al, 2022; Suzuki et al, 2013). Although the d-ROM test is not a specific indicator,

when it is elevated, we must check the patient's status very carefully.

MATERIALS AND METHODS

Patients and study design

We measured the d-ROM levels from the sera of 16 patients with active BP between 2017 and 2023. Written informed consent was obtained from all patients. The patients (7 males, 9 females; mean age = 76.7 years, range = 51−87 years) had been diagnosed on the basis of clinical and immunopathological criteria (Kershenovich et al, 2014). We recruited patients who had undergone blood tests before being treated with systemic corticosteroids. Patients who had received oral steroids from their previous physicians were excluded. We measured anti-BP180 and anti-BP230 antibodies in blood samples. We biopsied skin lesions and performed H&E staining on paraffin sections and DIF with IgG, IgM, IgA, and C3 on frozen specimens for diagnosis. We did not assay IgE antibodies. The following data were obtained from the patients' medical records: anti-BP180 antibody titers, anti-BP230 antibodies titers, and peripheral blood counts (white blood cell count, neutrophils, lymphocytes, and eosinophils). Complications that may affect d-ROMs values are listed. Serum d-ROM and BAP levels were evaluated before treatment and at 1 month of treatment (Tables 1 and 2).

Measurement of d-ROMs and BAP

The d-ROM levels and the BAP levels were measured using commercial kits and a reader (Redoxlibra, Wismerll, Japan). The d-ROM test procedure was as follows: (i) a serum sample is collected from the patients with BP; (ii) this sample is combined with a reagent from the d-ROM test kit, which contains a chromogenic substrate (N,N-diethylparaphenylenediamine) that changes color in the presence of oxidizing species; and (iii) the color change is then measured using the Free Radical Electron Evaluator (Redoxlibra) to quantify the levels of reactive oxygen metabolites in the sample. The normal range is 200−300 U.CARR.

The BAP test is similar to the d-ROM test: (i) a serum sample is obtained from the patient with BP; (ii) the blood sample is combined with a reagent from the BAP test kit, which contains a FRAP (ferric reducing ability of plasma) assay; and (iii) the degree of color change or reduction in the sample is then measured using a spectrophotometric device to quantify the BAP in the sample. The normal values are >2200 μM.

Eosinophil counts in H&E specimens

We counted the number of infiltrating eosinophils stained with H&E in a random 550 × 400-μm^2 under ×400 magnification. A total of 3 locations were measured: just below the blister, lateral to the blister, and randomly within the blister. We referenced a previous study (Izumi et al, 2016).

DIF staining for DiBrY

For this staining, we used frozen skin biopsy specimens from the erythematous nonblistering skin of patients with BP that were immediately frozen with liquid nitrogen and then stored at −80 °C. Samples were cut to a thickness of 4−5 μm, put on glass slides, dried, and soaked in PBS for 15 minutes. They were incubated with mouse anti-human DiBrY IgG (1:10 dilution) as the primary antibody at 37 °C for 60 minutes and were then stained with FITC-labeled goat anti-mouse IgG (Rockland, Limerick, PA) (1:500 dilution) as the secondary antibody. DAPI was used for nuclear staining at room temperature for 60 minutes. After washing and mounting, fluorescence microscopy was performed. Photomicrographs were taken with a Keyence BZ-X810 photomicroscope (Keyence, Osaka, Japan). DIF staining was performed on a total of 14 patients, excluding cases 11 and 14, because these specimens had been used up.

IHC staining for DiBrY

We used paraffin sections from blistering erythematous BP skin. These were deparaffinized and washed with PBS. The sections were blocked with Super Block (ScyTek Laboratories, Logan, UT) for 20 minutes. We used the mouse anti-human DiBrY (JaICA, Shizuoka, Japan) (1:10) mAb as the primary antibody and incubated for 60 minutes at room temperature or overnight at 4 °C. The secondary antibody was goat anti-mouse IgG biotin (Santa Cruz Biotechnology, Dallas, TX) (1:100) for 30−60 minutes and was conjugated to avidin horseradish peroxidase (eBioscience, Waltham, MA) (1:100) for 30−60 minutes. We visualized DiBrY with an avidin-biotinylated horseradish peroxidase (ABC) procedure followed by a metal-enhanced diaminobenzidine reaction (Dako, Glostrup, Denmark). Finally, the sections were counterstained with H&E. IHC staining was performed on a total of 16 patients.

BP180 chemiluminescent enzyme immunoassay and BP230 ELISA

All patients' serum samples were screened for IgG against BP180 NC16A and BP230. The sera were stored at −20 °C prior to analysis. Anti-BP180 NC16A antibodies were measured by chemiluminescent enzyme immunoassay (cutoff value = 9.0 U/ml) outsourced to SRL (Tokyo, Japan). Anti-BP230 autoantibodies were measured using a commercial BP230 ELISA kit (Medical and Biological Laboratories, Nagoya, Japan). The cutoff value was 9.0 U/ml for the chemiluminescent enzyme immunoassay for BP180 and was 5.0 U/ml for the ELISA for BP230.

The chemiluminescent enzyme immunoassay method utilizes a chemiluminescent substrate that produces light when the substrate interacts with an enzyme-labeled anti-human IgG antibody. The intensity of the emitted light is directly proportional to the amount of anti-BP180 antibody in the serum sample.

Statistical analyses

The d-ROM and BAP levels before treatment and at 1 month of treatment were compared using the paired *t*-test. A $P < .05$ was considered statistically significant. Pearson's correlation test was used to assess the relationship between the pretreatment d-ROM levels and each item (blood test results, BPDAI score, corticosteroid dosage, and the eosinophil count of the H&E specimen). Pearson's correlation test was used when the assumption of normality was not fulfilled. We considered $0.7 \leqq r \leqq 1.0$ to be a high correlation, $0.4 \leqq r < 0.7$ to be a moderate correlation, $0.2 \leqq r < 0.4$ to be a low correlation, and $0 \leqq r < 0.2$ to be no correlation. Statistical analyses were performed using the statistical software SigmaPlot, version 14.5 (Hulinks, Tokyo, Japan).

ETHICS STATEMENT

This study was approved by the Ethics Committee of Gifu University (number 2022-135). Written informed consent was obtained from all patients.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: NT, CT, HI; Data Curation: KT, CT; Formal Analysis: KT, CT; Investigation: KU, NK, HT, YY, DI, MM, NI, HN, YM, ES; Project Administration: HI; Writing - Original Draft Preparation: NT, CT; Writing - Review and Editing: CT, HI

DECLARATION OF ARTIFICIAL INTELLIGENCE (AI)/LARGE LANGUAGE MODELS (LLMS) USE

The authors declare no use of artificial intelligence/large language models in the preparation of this article.

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