

Increased detection of clinically significant antibodies and decreased incidence of delayed haemolytic transfusion reaction with the indirect antiglobulin test potentiated by polyethylene glycol compared to albumin: a Japanese study

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Background. The indirect antiglobulin test (IAT) can be potentiated by agents such as polyethylene glycol (PEG-IAT) and albumin (Alb-IAT). PEG-IAT is generally regarded as superior to Alb-IAT for the detection of clinically significant red blood cell (RBC) antibodies. However, supporting data come from Caucasian-dominant populations. Non-Caucasian populations should be investigated as well.

Material and methods. In this single-centre, retrospective, sequential study, Alb-IAT was used from 1989 to 1996 (8 years) and PEG-IAT from 1997 to 2008 (12 years). Pre-transfusion RBC alloantibody detection rates and specificity, post-transfusion alloantibody production, and the incidence of delayed haemolytic transfusion reaction were assessed and compared for the two periods.

Results. Although overall RBC alloantibody detection rates were comparable, PEG-IAT more frequently detected clinically significant antibodies such as anti-E, anti-Fy^b, and anti-Jk^a, and less frequently detected insignificant antibodies such as anti-Le^b and anti-P₁. New alloantibodies emerged comparably during the two periods. Delayed haemolytic transfusion reaction was less frequent during the PEG-IAT period (0.30% versus 0.12%, $p < 0.05$).

Conclusion. PEG-IAT was superior in the detection of clinically significant antibodies, reduced the detection of insignificant antibodies, and prevented delayed haemolytic transfusion reaction better than Alb-IAT among Japanese transfusion recipients in this retrospective survey of limited power.

Key words: indirect antiglobulin test, delayed hemolytic transfusion reaction, red cell antibody, polyethylene glycol.

Introduction

Delayed haemolytic transfusion reactions (DHTR) are adverse events¹ that occur 24 hours or more after red blood cell (RBC) transfusion. DHTR are often caused by irregular antibodies to antigens in the Rh, Kidd, Duffy or Kell blood group systems.

Transfusion of incompatible RBC may occur if a patient's antibody levels are below the detection threshold of a pre-transfusion test. As a consequence, specific B-cell clones proliferate, and their plasma cell progeny boost antibody titres to a detectable, and sometimes haemolytic level. This occurs mostly in

patients pre-exposed to foreign antigens through pregnancy or blood transfusion. To prevent the transfusion of incompatible RBC, sensitive methods of detecting RBC antibodies have been developed for pre-transfusion testing. These methods include the tube test, column agglutination technology, and microplate solid-phase systems, some of which may employ enzymatic antigen modification, indirect antiglobulin testing (IAT), and enhancement media such as albumin (Alb), low-ionic-strength solution, and polyethylene glycol (PEG).

The use of PEG-IAT, compared to Alb-IAT, has been shown to increase the detection rate of clinically significant irregular antibodies and decrease that of insignificant antibodies^{2,3}. A downward trend in the incidence of DHTR and an upward one in delayed serological transfusion reactions is most likely the result of adoption of the PEG-IAT (replacing Alb-IAT), as well as decreased lengths of stay⁴. However, these observations were derived from Caucasian-dominant populations. Other ethnic groups should be investigated as well. Our university hospital in Japan has used the tube test method. Alb-IAT was used until 1996 (hereafter, referred to as the Alb-IAT period) and PEG-IAT has been used since 1997 (hereafter, referred to as the PEG-IAT period). We previously reported that alloimmune response to erythrocyte antigens differs among Asian populations, and that the distribution of antibodies to these antigens differs from that of North American and European patients⁵. In the present study, we retrospectively compared clinical sequelae to RBC transfusion during the two periods described above in order to investigate changes in the incidence and specificity of detected irregular antibodies and to rank the efficacy of the tests in preventing DHTR. A subset of these data was published previously in Japanese⁶, but the present study revises earlier conclusions.

Materials and Methods

Patients

During the period when Alb-IAT was used for antibody screening and identification (January 1989 - December 1996), 31,086 patients were screened for antibodies and 4,651 recipients were transfused with 48,685 RBC bags, which were derived predominantly from whole blood donations of 200 mL (approximately 70%) or 400 mL (approximately

30%). During the period in which PEG-IAT was used for antibody screening and identification (January 1997 - December 2008), 40,887 patients were tested and 8,038 recipients were transfused with 60,661 RBC bags, which were derived predominantly from whole blood donations of 400 mL (approximately 70%), or 200 mL (approximately 30%).

Throughout both periods, there were no demographic trends, such as mass immigration, to alter the largely homogeneous Japanese population served by our institution. The current study includes more cases than previously reported⁶.

Assessment of clinical diagnosis of delayed haemolytic transfusion reaction

DHTR was determined according to the SHOT criteria⁷ and the report of Ness *et al.*⁸. Patients' transfusion-associated clinical symptoms, test results and blood transfusions during the two periods were analysed. DHTR was diagnosed if evidence of haemolysis attributable to transfusion, including jaundice, decreased haemoglobin, and impaired renal function, plus one or more of the following if confirmed between 24 hours and 90 days after transfusion: (i) a new alloantibody or increased titre of a previous known antibody; (ii) antibody elution from transfused RBC.

To avoid under- or over-diagnosis of DHTR or delayed serological transfusion reactions, a Certified Medical Technologist in Transfusion Medicine (accredited by the Japan Society of Transfusion Medicine and Cell Therapy) assessed the charts and laboratory results of all cases and consulted with the clinicians directly involved in the patients' care, as well as with medical and scientific staff of the transfusion department.

Pre-transfusion and post-transfusion irregular antibody test

Identical antibody tests were used for both pre-transfusion and post-transfusion testing during each period.

1) Albumin - indirect antiglobulin test

Polymerised bovine albumin solution (pH 7.2, protein concentration of 18.0-26.0%) from Ortho Clinical Diagnostics (OCD; NJ, USA) was used as the albumin reagent. After placing two drops

(approximately 100 μ L) of a patient's serum and a drop (approximately 50 μ L) of reagent red cells in a test tube, two drops (approximately 100 μ L) of the polymerised bovine albumin solution were added and mixed well. The mixture was incubated for 15 minutes at 37 °C. Rabbit polyspecific anti-human globulin reagent (anti-immunoglobulin and anti-C3d from OCD) was used until 1994, after which rabbit monospecific anti-human IgG (also from OCD) was used.

2) *Polyethylene glycol - indirect antiglobulin test*

Polyethylene glycol (20% [w/v], Sigma-Aldrich Corporation, St. Louis, MO, USA) prepared in-house⁹ was used for the modified PEG-IAT¹⁰. After placing two drops of a patient's plasma and a drop of reagent red cells in a test tube, two drops (instead of four) of PEG (approximately 100 μ L) were added and mixed well. The mixture was incubated for 15 minutes at 37 °C. Rabbit monospecific anti-human IgG (from OCD) has been used for PEG-IAT.

3) *Panel cells for antibody screening and identification*

Four cell sets have been used for antibody screening: Surgiscreen (three cells) and Di^a (one cell) (both from OCD). Resolve Panels A and B (from OCD) and PANOCELL-16 (from ImmucorGamma, Norcross, GA, USA), all with homozygous expression of most antigens, were used for antibody identification.

Statistical analysis

Statistical analysis was carried out using Fisher's exact test, a chi-square test (with/without Yate's correction) and the *t*-test, with $p < 0.05$ as the criterion for statistical significance. When appropriate, we used 95 percent confidence intervals (95% CI) to report different rates of antibody detection, antibody formation and DHTR incidences between the two groups. The software package used was StatMate III for Windows (ATMS, Tokyo, Japan).

Results

Detection rate and specificities of alloantibodies before transfusion

Table I shows the number of patients in whom alloantibodies were detected and the specificity of those alloantibodies. Among patients who underwent RBC antibody screening, irregular RBC antibodies

were found prior to transfusion in 351 (1.13%) during the Alb-IAT period and 511 (1.25%) during the PEG-IAT period. Although the detection rates were statistically indistinguishable ($p = 0.15$), the types of alloantibodies were significantly different between the two periods; PEG-IAT more frequently detected anti-E ($p < 0.001$), anti-Fy^b ($p < 0.05$), and anti-Jk^a ($p < 0.001$), and less frequently anti-P₁ ($p < 0.001$) and anti-Le^b ($p < 0.001$), as compared with Alb-IAT.

Detection rate and specificities of alloantibodies after transfusion

As shown in Table II, new alloantibodies were detected after RBC transfusion in 108 (0.85%) of 12,689 recipients, including 45 patients (0.97%) during the Alb-IAT period and 63 patients (0.78%) during the PEG-IAT period. Antibodies to antigens in the Kidd group system were newly detected during the PEG-IAT period more often than during the Alb-IAT period ($n = 15$, 0.19% versus $n = 3$, 0.06%), but this difference was not statistically significant ($p = 0.08$). Rh system antibodies were detected in 0.65% of the recipients in both periods. Although antibodies to antigens in the MNSs system were detected less frequently during the PEG-IAT period than during the Alb-IAT period, this trend was not statistically significant ($p = 0.06$).

Number of recipients with delayed haemolytic transfusion reaction

DHTR was observed in 24 (0.19%) of 12,689 RBC recipients as shown in Table III. The overall incidence of DHTR during the Alb-IAT period was 0.30% (14 cases in 4,651 recipients), which was significantly higher than during the PEG-IAT period (0.12%, 10 cases in 8,038 recipients; $p = 0.03$). The difference in incidence between the two periods was significant statistically among male recipients, with a DHTR incidence of 0.28% during the Alb-IAT period and 0.05% during the PEG-IAT period ($p = 0.02$). There was no significant difference in the incidence of DHTR in relation to the age of recipients or detection day interval after transfusion.

Three recipients, including two during the Alb-IAT period (cases 11 and 14) and one during the PEG-IAT period (case 18), exhibited DHTR as a primary immune reaction without previous known alloantigen exposure through blood transfusion or pregnancy.

Table I - Antibody specificity detected by two IAT screenings.

		Alb-IAT period	PEG-IAT period	p value
Number of cases studied		31,086	40,887	-
Number of case in which RBC antibodies were detected (%)		351 (1.13%)	511 (1.25%)	0.15
	[95% CI]	[1.01-1.25]	[1.14-1.36]	
Antibody specificity by blood group system				
Rh system	D	8	15	0.42
	C	5	11	0.33
	c	27	35	0.95
	E	98	208	<0.001
	e	3	8	0.29
Duffy system	others	2	2	0.78
	Fy ^a	0	0	-
	Fy ^b	7	23	<0.05
Kidd system	Jk ^a	2	23	<0.001
	Jk ^b	3	4	0.99
Kell system	K	1	1	0.85
	others	1	3	0.46
Diego system	Di ^a	15	31	0.15
	Di ^b	0	0	-
P system	P _I	50	28	<0.001
MNS system	M	25	23	0.21
	N	0	0	-
	S	5	10	0.44
	s	0	0	-
	Others	2	0	-
Lewis system	Le ^a	111	135	0.54
	Le ^b	32	7	<0.001
Other systems		14	18	0.95
Not identifiable		2	4	0.63
Total antibodies		413	589	

Table II - Number of new specificities by blood group system detected by IAT within 90 days after RBC transfusion and characteristics of the cases in the two periods.

		Alb-IAT period	PEG-IAT period	p value
Number of RBC transfusion recipients		4,651	8,038	
Number of cases of new antibody (%)		45 (0.97%)	63 (0.78%)	0.28
	[95% CI]	[0.69-1.25]	[0.59-0.98]	
Median interval between detection of antibody and transfusion*, in days		27	25	0.91
Antibody specificity by blood group system				
Total number of antibody specificities (specificity by blood group)		64	85	
Rh system		30	52	0.99
Duffy system		3	2	0.54
Kidd system		3	15	0.08
Kell system		0	0	-
Diego system		2	1	0.63
P system		4	1	0.12
MNS system		6	2	0.06
Lewis system		6	5	0.36
Other systems		9	5	0.06
Autoantibody		1	2	0.63

*Since first transfusion in study period.

Table III - Characteristics of DHTR in the two periods

	Alb-IAT Period	PEG-IAT Period	p value
Number of DHTR cases (%)	14 (0.30%)	10 (0.12%)	0.03
[95% CI]	[0.14-0.46]	[0.05-0.20]	
Gender (%)			
Male	7(0.28%)	3(0.05%)	0.02
[95% CI]	[0.07-0.48]	[0.01-0.11]	
(Total recipients)	(2,541)	(5,530)	
Female	7(0.33%)	7(0.28%)	0.75
[95% CI]	[0.09-0.58]	[0.07-0.49]	
(Total recipients)	(2,110)	(2,508)	
Age (years, median)	62	49	0.26
With no previous immunization*	2	1	0.63
Interval between detection of antibody and transfusion (days, median)	12	12.5	

*Transfusion or pregnancy history before transfusion.

Test results from recipients with delayed haemolytic transfusion reaction

Test results for individuals with DHTR are shown in Table IV. Antibodies to antigens in the Rh and Kidd blood group systems were the most frequently detected RBC alloantibodies among patients with DHTR; antibodies to Rh antigens were detected in eight patients (cases 1-8) during the Alb-IAT period and in eight patients (cases 15-21, and 24) during the PEG-IAT period. Antibodies to antigens in the Kidd blood group system were detected in two patients (cases 8 and 13) during the Alb-IAT period and in five patients (cases 20-24) during the PEG-IAT period. DHTR-related antibody was detected in post-transfusion RBC eluates in six (43%) of 14 patients during the Alb-IAT period and nine (90%) of ten patients during the PEG-IAT period.

In this study, the median antibody detection interval after transfusion was 12 days in the Alb-IAT period (range, 2-63 days), similar to the 12.5 days in the PEG-IAT period (range, 7-39 days).

Discussion

DHTR, like acute haemolytic transfusion reactions, have more consequences than delayed serological transfusion reactions⁸, which simply show a positive direct antiglobulin test without any clinical sequelae such as haemolysis, hepatic and/or renal disturbance). Although many transfusion specialists and biomedical

companies have studied various antibody testing methods with more sensitivity, including column agglutination technologies, solid-phase RBC adherence assays, fluorescence cytometry^{11,12}, even now DHTR persists as one of the serious hazards of transfusion^{3,4,13,14}. Among Japanese transfusion services, 65% reported using the tube test method compared to 37% which used agglutination technologies. IAT enhancements of the tube test method include low ionic strength saline (35%), PEG (31%), and bovine albumin/polymerised bovine albumin (31%)¹⁵. To avoid false negative results in hyperglobulinemia, we use a modified PEG-IAT¹⁰ with equivalent sensitivity; the modification consists of using two drops of PEG instead of four drops as originally described⁹.

The present survey among Japanese patients shows that, compared to Alb-IAT, PEG-IAT can detect clinically significant antibodies, including anti-E, anti-Fy^b and anti-Jk^a, with better detection sensitivity before RBC transfusion (Table I). Moreover, post-transfusion investigations show a trend in favour of PEG-IAT over Alb-IAT for detecting antibodies to antigens in the Kidd blood group system (0.19% versus 0.06%, $p=0.08$) (Table II). Furthermore, the incidence of DHTR was significantly lower during the PEG-IAT period (0.12%) than during the Alb-IAT period (0.30%). In a prior study⁶, our group concluded that the frequency of DHTR was not statistically

Table IV - DHTR-associated alloantibodies by case and period

(A) Alb-IAT period						
Case no.	Gender	Newly acquired alloantibodies in serum after transfusion	Detection of alloantibodies before transfusion	DAT ⁽¹⁾	Interval between RBC transfusion and antibody detection (days)	Comments
1	M	anti-E		+	63	Eluate: anti-E +anti-c
2	F	anti-E	anti-Di ^a +pan ⁽²⁾	+ ⁽⁴⁾	10	
3	F	anti-E+c		+	46	Eluate: anti-E+pan ⁽²⁾
4	M	anti-c	anti-Fy ^b +E+Di ^a	+	10	Eluate: anti-c+pan ⁽²⁾
5	F	anti-c	anti-E	negative	79	
6	F	anti-C+e+Di ^a		+	6	Eluate: anti-pan ⁽²⁾
7	M	anti-E+c+Fy ^b + M		negative	14	
8	M	anti-e+Jk ^a +P1 ⁽³⁾	anti-C	negative	2	2 episodes of DHTR
9	M	anti-S+pan ⁽²⁾	anti-E	+	12	Eluate: anti-S
10	F	anti-S	anti-Chido+Di ^a +pan ⁽²⁾	+ ⁽⁴⁾	53	
11	F	anti-S+(P1)) ⁽³⁾	anti-Di ^a by PEG-IAT	+	15	Eluate: anti-S
12	M	anti-M		negative	2	
13	F	anti-Jk ^b		negative	12	
14	M	anti-Di ^a +NI ⁽⁵⁾		negative	5	
(B) PEG-IAT period						
15	F	anti-E		+	8	Eluate: anti-E
16	F	anti-E		+	39	Eluate: anti-E+pan ⁽²⁾
17	F	(-)		+	27	Eluate: anti-E ⁽⁶⁾
18	M	anti-E+c		+	16	Eluate: anti-E+pan ⁽²⁾
19	F	anti-E+pan ⁽²⁾		+	8	anti-HLA Eluate: anti-pan ⁽²⁾ DHTR and hyperhaemolysis
20	F	anti-E+c+Jk ^a +Bg ^a +Bg ^c		negative	14	Two haemolytic episodes (anti-E+c+Jk ^a , and anti-Bg ^a +Bg ^c)
21	F	anti-E+c+Jk ^a +S+Bg ^a	anti-pan ⁽²⁾	+ ⁽⁴⁾	11	Eluate: anti-pan ⁽²⁾ DHTR and hyperhaemolysis
22	M	anti-Jk ^a		+	10	Eluate: anti-Jk ^a
23	M	anti-Jk ^a		+	7	Eluate: anti-Jk ^a
24	F	anti-Jk ^a		+	17	Eluate: anti-E ⁽⁶⁾ +Jk ^a

⁽¹⁾ Direct anti-globulin test; ⁽²⁾ Pan-agglutinating antibody; ⁽³⁾ Reaction only in saline; ⁽⁴⁾ DAT positive before RBC transfusion; ⁽⁵⁾ Unidentified antibody; ⁽⁶⁾ Reaction only in eluate of patient's cells after RBC transfusion.
Case 20 has been reported previously (reference 34).

different between the Alb-IAT (0.24%) and PEG-IAT (0.13%) periods. This difference from the present study was potentially due to a smaller sample size: 4,158 recipients during the Alb-IAT period and 4,651 during the PEG-IAT period⁶. We now estimate that 7,602 patients (two-sided) would need to undergo the new screening method (PEG-IAT) for the comparison with the standard Alb-IAT in order to rule out an

absolute difference of 0.11% in DHTR between the Alb-IAT and PEG-IAT periods. The calculated sample size assumes a type 1 error rate of 5%. In our previous work⁶, the sample size (4,651) for PEG-IAT was smaller than necessary, and led to the conclusion that there was no significant difference in the incidence of DHTR between the two methods.

It is difficult to explain why the incidence of DHTR

during the two periods differs for men, but not for women as shown in Table III. National demographics suggested that a majority of women over about 35 years old have been exposed to alloantigens through pregnancy. Immunological responders may have acquired alloantibodies and such antibodies may be boosted by later transfusion¹⁶.

Taken together, Alb-IAT may have failed to detect some significant antibodies before transfusion and even after DHTR. For example, Alb-IAT detected anti-S+(P₁), with the P₁ in parentheses representing a reaction only in physiological saline, in patient n. 15 in Table IV. However, when PEG-IAT was used afterward, anti-Di^a was also detected. Thus, Alb-IAT failed to detect anti-Di^a because the antibody level was below the detection limit. Consequently, screening using PEG-IAT is better at preventing DHTR.

Given the gradual shift of donation blood volume from 200 mL to 400 mL over two decades, the average number of transfused bags (donor exposure) per recipient was different, 10.5 bags/recipient in the Alb-IAT period versus 7.5 bags/recipient in the PEG-IAT period. Changes in the number of RBC can alter the risk of developing the antibodies which cause DHTR or delayed serological transfusion reactions. Fewer donor exposures per patient may reduce alloantigen exposures, resulting in fewer primary and/or secondary immune responses. If so, the incidence of DHTR and delayed serological transfusion reactions would be lessened⁴.

DHTR is mostly caused by antibody titre rebound in a secondary immune response, and rarely by a primary immune response¹⁷. Redman *et al.* conducted a 9-month follow-up study of 452 immunocompetent surgical recipients, and reported that the mean time to antibody detection after transfusion was 57 days in 16 patients without a history of blood transfusion or pregnancy¹⁸. Ishimaru *et al.* reported a patient with anti-C + e + Jk^a who developed a primary immune response-induced DHTR 22 days after transfusion¹⁹. The causal anti-Jk^a could be detected using PEG-IAT and Capture-R Ready-Screen from 24 days after transfusion, but was not detected with Alb-IAT during the 108-day study period.

Pan-agglutinating autoantibodies also developed concomitantly with alloantibodies after RBC transfusion in many DHTR cases. DHTR patients n.

2, 10 and 21 had pan-agglutinating autoantibodies before transfusion, and positive direct antiglobulin tests with no symptoms of autoimmune haemolytic anaemia (AIHA). DHTR patients n. 19 and 21 developed hyperhaemolysis²⁰ despite transfusion of antigen-negative RBC compatible with cognate alloantibodies. Allogeneic transfusion can be a risk factor for autoimmune-associated events, as reported in some patients with elevated titres of pan-agglutinating autoantibodies after transfusion²¹⁻²³.

Alloantibodies were eluted from circulating RBC in six (43%) of 14 DHTR patients during the Alb-IAT period and in nine (90%) of ten patients during the PEG-IAT period. Autoantibodies were detected concomitantly with alloantibodies in three (21%) of 14 patients during the Alb-IAT period and four (40%) of ten patients during the PEG-IAT period. Pan-agglutinating autoantibodies developed concomitantly with Rh system in seven DHTR recipients (cases 3, 4, 6, 16, 18, 19 and 21). In contrast, most patients (83 of 108) with new alloantibody formation did not develop DHTR or produce autoantibodies. Only one patient with autoantibody before transfusion and anti-E formation after transfusion did not manifest haemolysis. This may indicate that preformed/newly formed autoantibody can be a risk factor for or an enhancer of haemolysis.

Although the precise mechanism of autoimmunisation in association with alloimmunisation is unknown, pathogenic auto-antibodies and activated autoreactive CD4⁺ T helper 1 cells are reported in most AIHA patients to be specific for Rh proteins on the RBC membrane²⁴. Ahrens *et al.* speculated that the antibody would initially be of low affinity, and would not, thus, affirmatively bind to alloantigen epitopes, but cross-react to common structures like true autoantibodies²⁵. Our survey supports this view. Additionally, antibodies from most patients with AIHA react with conformational epitopes constituted by RhD- or cE-specific exofocal epitope loops²⁶. A later hypothesis suggests that non-exofacial polymorphisms within cytoplasmic or transmembrane domains in RBC contribute to immunogenicity of antigens and stimulation of T cells for autoantibody development²⁷. Thirdly, it has been suggested that perhaps AIHA sometimes occurs because lymphocytes, from transfusion, survive and create a "graft-versus-host" scenario in which the donor

lymphocytes produce alloantibody that sensitises the host's RBC, and thus pseudo-autoantibody²², or passenger lymphocyte syndrome^{28,29}. As all blood/cellular components for transfusion have been irradiated at 15 Gy since 1989 in our hospital³⁰, the possibility of "alloantibody formation" by transfused lymphocytes is very rare, although no fresh frozen plasma is irradiated.

It was reported that PEG increases the rate of detection of autoantibodies, which are not of clinical relevance for transfusion purposes³¹. To avoid detecting less significant autoantibodies, many transfusion services do not use an autologous control test with auto-RBC and auto-serum. When a haemolytic adverse reaction is observed, however, an autologous control should be performed because it provides meaningful information for verification steps, including reactivity for the direct antiglobulin test even when no antibodies can be detected in recipients' sera.

It is difficult to diagnose DHTR in some patients. Most of the delayed reactions in this survey were identified in retrospect, but not diagnosed at the time they occurred. Even when findings are consistent with DHTR, they can be masked by or attributed to underlying hepatobiliary/renal diseases and pharmaceutical side effects. Future investigations should include haemolysis work-ups specific to blood transfusion, such as immunoglobulin class/subclass determination and the monocyte monolayer assay³²⁻³⁴.

Although retrospective studies have limited power for drawing conclusions, the authors consider that PEG-IAT, having a higher sensitivity than Alb-IAT, was provably more effective in the detection of significant alloantibodies and in the prevention of DHTR among a Japanese population, as it is among Caucasian-dominant populations.

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