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Neutralizing-enhancing monoclonal antibody recognizes the denatured glycoprotein of viral haemorrhagic septicaemia virus

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Summary. Two monoclonal antibodies (MAbs), 1H10 and 1F10, have been selected against the denatured glycoprotein of the viral haemorrhagic septicaemia virus (VHSV) of salmonids by immunoblotting. Three reference VHSV serotypes (F1, F2, 23.75) and five VHSV isolates from either different host species (trout, salmon, barbel) or geographical locations in Spain reacted with both MAbs by ELISA. In vitro neutralization of all VHSV serotypes but not infectious haematopoietic necrosis virus (IHNV) was obtained only with MAb 1H10. However, when the MAb 1H10 (but not the non-neutralizing Mab 1F10) concentration was below 100 µg/ml rather than neutralizing VHSV infectivity, plaque counts increased 2–3-fold. MAb 1H10 is unique in that it has both, an in vitro enhancing infectivity effect (not described yet for VHSV) and it recognizes denatured G protein in contrast to other previously described neutralizing MAbs against VHSV.

Introduction

Viral haemorrhagic septicaemia (VHS) is a disease of salmonid fishes causing severe damage in fish farms in Europe [23]. The viral haemorrhagic septicaemia virus (VHSV) is a membrane-enclosed negative strand RNA rhabdovirus of the genus *Lyssavirus*. Its five virion proteins are designated, L (the RNA-dependent RNA polymerase of 150–200 kDa), N/Nx (the phosphorylated nucleoprotein of 45–50 kDa) [3], M_1 and M_2 (the matrix proteins of 22 and 28 kDa) and G (the glycoprotein that carries neutralizing epitopes of 65–80 kDa) [8].

The few reported VHSV neutralizing MAbs have not yet allowed the complete identification of the molecular characteristics of their target viral epitopes [12, 13]. For instance, the G epitope(s) defined by in vitro neutralizing anti-G MAbs [12, 13; Thiry, pers. comm.] are disulphide-bonds dependent and deglycosylation does not affect their recognition. However, whereas one of the in vitro neutralizing anti-G MAb defining epitope(s) dependent on disulphide

bonding (β-mercaptoethanol sensitive) neutralized VHSV in vivo, other in vitro non-neutralizing anti-G MAb defining epitope(s) independent of disulphide bonding also neutralized VHSV in vivo [13].

In the experiments reported here we describe an in vitro neutralizing MAb defining an epitope(s) which is recognized under denaturing conditions (independent of disulphide bonding) which causes in vitro enhancement rather than neutralization of viral infectivity when used at low concentrations. Although it is not yet known whether or not the epitope defined by this neutralizing-enhancing MAb is recognized by the neutralizing antibodies (Abs) of salmonid fish, the results obtained suggest further studies to define whether or not subunit vaccines should contain this (these) epitope(s).

Materials and methods

Viruses

The strains of virus used were VHSV-F_1 and VHSV-F_2, provided by Dr. P. E. Vestergard-Jorgensen (National Veterinary Lab, Arhus, Denmark); VSHV-23.75, provided by Dr. P. De Kinkelin (INRA, Centre Recherche Jouy en Josas, France); and IHNV-Cedar strain provided by Dr. R. Hedrick (University of California, Davis, California, U.S.A.). The five VHSV isolates from Spain, were obtained from: rainbow trout, *Oncohrynchus mykiss* Richardson (689, from Galicia in 1984; 471, from Navarra in 1986; and 144, from Salamanca in 1984), Atlantic salmon, *Salmo salar* Linneus (472, from Cantabria in 1986) and barbel, *Barbus graellsi* Steindachner (798, from Aragón in 1986). Unless otherwise indicated, the Spanish isolate VHSV-144, was used throughout the experiments. The protein bands of VHSV isolate 144 were identified by (1) Coomassie blue staining of polyacrylamide gels of purified virus, (2) [^{35}S]methionine labeled viral proteins, and/or (3) immunoprecipitation [1, 2] with international reference polyvalent antisera (gift Dr. P. De Kinkelin). All protein bandings were compared to those obtained with the reference serotypes mentioned above [1, 2].

Virus propagation, purification and titration

Viruses were propagated in the epithelioma papillosum cyprini (EPC) cell line as described before [2, 3]. Cell culture techniques (RPMI-1640 medium; Flow, Ayrshire, Scotland) and purification of VHSV were essentially those of De Kinkelin [8] as modified by Basurco and Coll [2] and Basurco et al. [3].

Monoclonal antibodies

Three female mice (BALB/c) were given 9 intraperitoneal injections of 25 μg of polyethyleneglycol (PEG) concentrated VHSV over 9 months (1 per month). The ascites from one of the mice contained polyclonal Abs against all the proteins of VHSV as shown by immunoblotting and was able to neutralize VHSV infectivity. MAbs from this mouse against VHSV were obtained as described before and were detected by indirect enzyme linked immunosorbent assay (ELISA) [2, 14]. Spleen cells from the immunized mice were fused with the myeloma cell line P3-X63-Ag 8653 as described [14]. Fusion, cloning procedures (twice by limiting dilution), and cultivation of hybridoma cells were performed as previously described [3, 19]. The isotype of MAbs was determined by ELISA (Biorad kit for isotype mous MAb determination; Biorad, Richmond, Virginia, U.S.A.). Ascites production was obtained by injection of physiological saline [7]. The ascites were clarified by centrifugation

at 3000 g 10 min and stored at −40 °C until used. The ascites samples were precipitated by 50% of saturation of ammonium sulphate, electrophoresed in 4–20% polyacrylamide precast gels (Biorad), and the gels stained with Coomassie blue. Relative concentration of the IgG was estimated by comparing the intensity of the IgG stained bands with standards [3, 19].

Neutralizing MAb 5G3 against IHNV was a gift of Dr. J. Winton (Seattle, Washington, U.S.A.).

Immunoblotting

The protein bands were transferred from 10–15% polyacrylamide gradient gels to nitrocellulose membranes, [3]. After blocking with dilution buffer (0.5% bovine serum albumin, 0.1% Tween-20, 0.01% merthiolate, 50 mg/l phenol red in 10 mM sodium phosphate, 0.15 M sodium chloride, pH 7.6), the strips were incubated 1 h with the hybridoma supernatants diluted 3-fold in dilution buffer. To identify the bands, some strips were stained with Amido-black stain and the remainder of the gel was stained with Coomassie blue. The bands were developed with 1 mg/ml diaminobenzidine in low background citrate buffer [3].

Purification of viral proteins

VHSV proteins were isolated by preparative gel electrophoresis of purified [^{35}S]methionine labeled VHSV in the presence of SDS and β-mercaptoethanol. Preparative electrophoresis was performed in a 15% polyacrylamide separating gel. The bands were visualized during electrophoresis using the green dye Chromaphor (Promega Madinson, WI, USA). After electrophoresis, the bands were identified by autoradiography as described [1, 2], and removed from the gel approximately at the following molecular weights, 60–80 kDa for G, 40–45 kDa for N, 30–40 kDa for Nx, 20–25 kDa for M_1 and 15–20 kDa for M_2. The bands were cut in small pieces and transferred to a dialysis bag containing 2 ml of 0.2 M Tris/sodium acetate, pH 7.4 with 1% SDS. The proteins were electroeluted in 0.05 M Tris sodium acetate, pH 7.4 with 0.1% SDS at 100 V/100 mA for 12 h until the green dye was in solution. After electroelution, the bags were placed into 50 volumes of 0.2 M ammonium bicarbonate for further dialysis. The protein solution was removed from the bags, lyophilyzed and resuspended in 50 µl of distilled water. Precipitates were discarded after centrifugation at 10 000 g for 15 min. To confirm isolation and purity, the isolated proteins were reelectrophoresed and stained by silver nitrate (Biorad Silver staining Kit, Richmond, Virginia, U.S.A.), then dried onto cellophane and autoradiographed using X-ray film (Kodak X-Omat Xar-2). Purity, as roughly estimated by densitometry of the silver nitrate stained gels following reelectrophoresis of the isolated proteins was higher than 80%. Protein concentration was first estimated by absorbance at 280 nm, using ε of 1.4, and that value was then used to calibrate the silver nitrate staining estimate. Protein content was adjusted to 200–300 µg per ml, and proteins were kept frozen at −20 °C until used.

Neutralization tests

For initial screening of neutralizing MAbs, 100 tissue culture infectious doses ($TCID_{50}$) of VHSV in 50 µl of culture medium were incubated with 50 µl of supernatant from each of the hybridomas for 2 h at 14 °C. Then, 50 µl of each mixture was transferred to a well of a 96-well plate containing monolayer cultures of EPC cells. Controls included cultures containing no supernatants or polyclonal mouse ascites with neutralizing activity diluted 10-fold. Cultures were incubated at 14 °C and examined daily for cytopathic effect.

To confirm the presence of neutralizing activity, plaque neutralization tests were prepared in 24-well plates, with overlays of 0.8% ultra-low gelling temperature agarose (Sigma

Chemical Co., St Louis, MO, U.S.A.). VHSV (200–300 µl) and MAbs (100–200 µl) were mixed and incubated overnight at 14 °C. Then the mixtures were absorbed to EPC monolayers at 14 °C 1 h, washed, overlaid with 400 µl of 0.8% low-melting point agarose per well and incubated at 14 °C for 4–5 days. Plaque forming units (PFU) were estimated and results were expressed by the following formula, number of plaques in the presence of Ab/number of plaques in the absence of Ab × 100.

To calculate neutralization indices ($\log_{10}$ NI) antibody was precipitated from ascites with 50% saturated ammonium sulphate (final protein concentrations adjusted to 300 µg/ml), dialysed and then incubated at 14 °C overnight with serial 10-fold virus dilutions (10^{-3} to 10^{-10} $TCID_{50}$) in quadruplicate. Then 100 µl of the mixture was added to 100 µl of EPC monolayers in 96 well cultures and plates incubated for 3 days at 14 °C. Neutralization indices (NI) were calculated by the following formula,

$$-\log (TCID_{50} \text{ of virus control} - TCID_{50} \text{ of tested Ab}).$$

Results

Identification of the specificity of the MAbs

The target antigens of the MAbs were identified by immunoblotting against the electrophoretically separated proteins from PEG-concentrated VHSV (Fig. 1), so that the Nx protein could be present [3]. The bands were identified as virally induced, G, N, Nx, M_1 and M_2 by comparison of ^{35}S-labeled protein patterns from infected cells with non-infected controls [1, 2]. Of 19 MAbs selected by indirect ELISA, only 7 were capable of binding to their denatured target proteins and only 2 were specific for the G protein. None of these MAbs reacted with cellular control antigens. MAb 1H10 (IgG_1 K) reacted with a 58 kDa region coincident with the ^{35}S-labeled and the Coomassie blue stained band identified as G wherease MAb 1F10 (IgG_{2b} K) reacted with some minor component faintly stained by Coomassie blue at about 65 kDa. The different

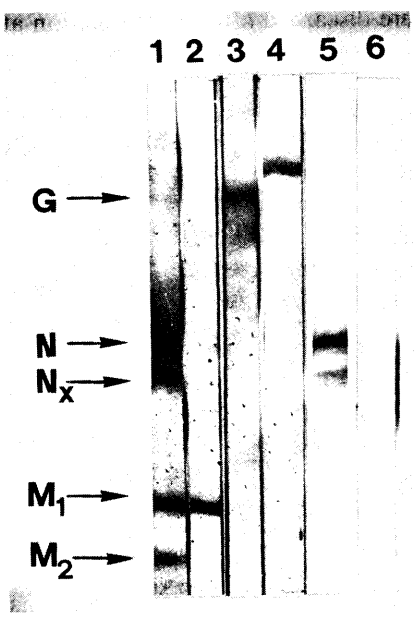

Fig. 1. Recognition of VHSV proteins with anti-VHSV monoclonal antibodies by immunoblotting. Concentrated VHSV virus was electrophoresed, transferred to nitrocellulose, incubated with 3-fold diluted hybridoma supernatants and developed. Viral proteins (G, N/Nx, M_1, M_2) were identified by Coomassie blue staining of the nitrocellulose filter and by autoradiography of [^{35}S]methionine-labeled VHSV (arrows). *1* Polyclonal ascites anti-VHSV raised in mice prior to fusion; supernatants from anti-VHSV selected hybridomas, *2* 1C10, anti-M_1; *3* 1H10, anti-G; *4* 1F10; anti-G; *5* 2D5, anti-N/Nx; *6* supernatant from non-producer hybridoma

degree of glycosylation of G might account for the varying molecular weight [13].

Confirmation of the immunoblotting results was obtained by ELISA using plates coated with VHSV proteins (G, N, Nx, M_1 and M_2) purified by preparative electrophoresis and isolated by electroelution. Under those conditions, a low but significant reaction was obtained also between MAb 1H10 and M_2 (not shown). Specificity of 1H10 was further confirmed in another laboratory by ELISA using plates coated with non-denatured purified protein G (about 2 ng/well sensitivity) (not shown).

Recognition of VHSV serotypes by the anti-G MAbs

Table 1 shows the reactivity of 5 VHSV isolates from Spain, and 3 VHSV reference serotypes (as defined by seroneutralization with polyclonal Abs) with the 2 anti-G MAbs. The MAbs reacted similarly with all the VHSV reference serotypes. No strong differences were detected in their reactivity with the anti-G MAbs among the VHSV isolates made in different fish species, barbel (798), rainbow trout (471, 689, 144) and salmon (472).

To test for variability of the G epitope defined by MAb 1H10, we used it at two neutralizing concentrations. At the low neutralizing concentration of 300 µg/ml, neutralization indices of 0, 1.8, 1.7 and 1.2 were obtained for IHNV, VHSV-F_1, VHSV-F_2 and VHSV-23.75, respectively. Under the same experimental conditions neutralizing MAb 5G3 against IHNV showed a neutralization index of 2.5 (Table 2). At the high neutralizing concentration of 2000 µg/ml, MAb 1H10, reduced 80–100% of the number of plaques of all VHSV reference strains F_1, F_2 and 23.75 (and the 5 VHSV isolates from Spain) but not IHNV (not shown).

Table 1. Reaction of anti-G MAbs with the VHSV isolates from Spain [1, 2] and the three reference VHSV serotypes

MAbs	Viruses							
	798	471	472	689	144	F_1	F_2	23.75
1F10	1.6	0.8	0.7	0.6	1.8	0.6	1.8	1.0
1H10	1.2	0.7	0.6	0.9	1.2	0.6	0.8	1.0

Spanish virus isolates were from *Barbus graellsi* (798), *Onchorynchus mykiss* (471, 689, 144) and *Salmo salar* (472). The ELISA assays were performed by using 3 µg of purified virus per well. The supernatants of the hybridomas were diluted 20-fold in dilution buffer so as to produce an ELISA titre of the same order of magnitude. The results were finally normalized by the ELISA absorbances in parallel with the use of rabbit polyvalent antisera (F_1 + F_2 + 23.75) as an international reference standard (gift of Dr. De Kinkelin). The correction factor varied between 1–2-fold. The results were similar whether rabbit anti-VHSV 144 antisera or prefusion mouse anti-VHSH ascites were used for normalization. Background values were subtracted from all the data

Table 2. Neutralization indices of VHSV serotypes and IHNV by Abs to VHSV and IHNV

Viruses	Anti-IHNV 5G3	Anti-VHSV		
		polyclonal	1F10	1H10
IHNV Cedar	2.5	ND	−0.5	0
VHSV F_1	−0.2	0.8	0.3	1.8
VHSV F_2	−0.2	0.5	0	1.7
VHSV 23.75	−0.2	1.2	0	1.2

All the Abs used were mouse ascites concentrated by ammonium sulphate except the anti-IHNV which was used as concentrated supernatant from hybridoma 5G3 (gift of Dr. J. Winton). Results are expressed as $\log_{10}$ neutralization index ($\log_{10}$NI)

ND Not determined

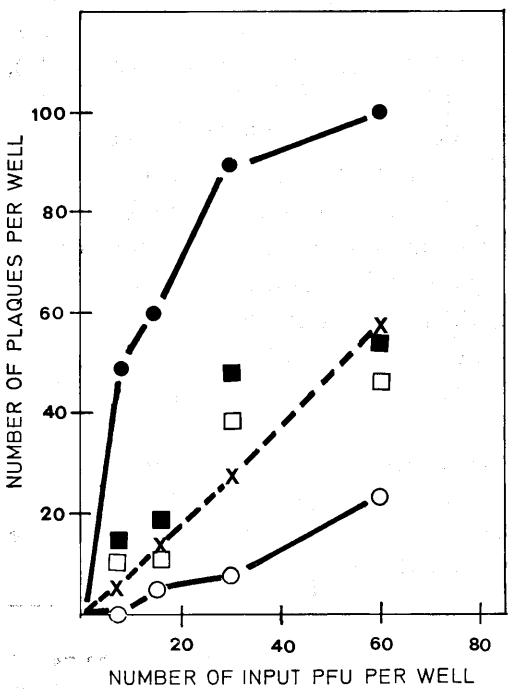

Fig. 2. Number of plaques obtained after incubating VHSV with hybridoma supernatants against VHSV. VHSV at 8×10^4 PFU/ml (300 µl) and Abs (100 µl) were mixed and incubated overnight at 14 °C. Then the mixtures were absorbed to EPC monolayers at 14 °C 1 h, washed, overlaid with 400 µl of 0.8% low-melting point agarose per well and incubated at 14 °C 4–5 days. Mixtures contained: ×–× no Abs; ○–○ polyclonal ascites with anti-VHSV raised in mice prior to fusion; ●–● supernatant from anti-G MAb 1H10; ■ supernatant from anti-G MAb 1F10; □ supernatant from anti-N MAb 2D5

Enhancement of infectivity of VHSV

Microneutralization tests performed during the screening of 500 hybridomas gave non-reproducible results depending on the relative amounts of the hybridoma culture supernatants and the VHSV (100 $TCID_{50}$/well). Therefore, neutralization was performed by plaque reduction with the two anti-G MAbs (1H10 and 1F10) identified by immunoblotting. Most of the supernatants tested from the hybridomas did not change the number of plaques (Fig. 2 and Table 3), except one from 1H10 which showed about a 2–3-fold increase and the mouse

Table 3. Enhancement of VHSV infectivity

Source of Abs	Protein (μg/ml)	PFU (% of control)
Supernatant anti-G MAb 1H10	30	196 ± 72 (n = 5)
Ascites anti-G MAb 1H10	500	27 ± 6 (n = 2)
IgG anti-G Mab 1H10	50	140 ± 10 (n = 2)
IgG anti-G Mab 1H10	300	12 ± 12 (n = 2)
Supernatant anti-G MAb 1F10	50	106 ± 28 (n = 4)
Ascites anti-G MAb 1F10	500	103 ± 20 (n = 3)
IgG anti-G MAb 1F10	50	100 ± 10 (n = 2)
IgG anti-G MAb 1F10	300	100 ± 12 (n = 2)
Mouse ascites polyclonal	500	33 ± 11 (n = 2)

Neutralization was performed by the plaque reduction test. Average ± SD from different experiments (n) are represented. Number of plaques in the control (absence of tested Ab) varied between 30–65 PFU per well. IgG anti-G was obtained by ammonium sulphate concentration of the ascites

% of control = (number of plaques in the presence of tested antibody / number of plaques in the control) × 100

Mouse ascites polyclonal: polyclonal ascites anti-VHSV raised in mice prior to fusion

polyclonal ascites which reduced the number of plaques approximately in half. Enhancement of the number of plaques was seen with supernatants from the anti-G MAb 1H10 but not with the anti-G Mab 1F10 (Fig. 2). Because some neutralizing Abs might increase the number of plaques when used at subneutralizing concentrations [4, 5, 16], MAbs 1H10 and 1F10 were obtained as ascites from mice and then used at higher concentrations in neutralization assays. Total neutralization of VHSV was obtained only with MAb 1H10. Enhancement of the number of plaques was seen at subneutralizing concentrations of the neutralizing anti-G MAb 1H10 but not with the non-neutralizing anti-G MAb 1F10. The enhancement was seen in different experiments (Table 3), but always at subneutralizing concentrations of anti-G MAb 1H10 ($< 100\,\mu g$ per ml). If the virus/MAb 1H10 mixture was not washed after the 1 h absorption but remained during the 3-day incubation, the enhancement effect was seen over a wider range of MAb concentrations ($\leqslant 150\,\mu g/ml$) and a higher concentration of MAb was needed to obtain a comparable neutralization effect as when the virus/MAb 1H10 was washed immediately after the 1 h absorption (not shown).

Discussion

This paper describes the properties of a unique MAb which reacts with the G protein of VHSV in Western blots and enhances virus in vitro infectivity 1.5–3-fold at subneutralizing concentrations.

All neutralization epitopes of VHSV described to date are dependent on conformationally dependent disulphide bonds [12, 13]. The epitope recognized

by MAb 1H10 is, however, resistant to denaturing agents as shown by immunoblotting. The availability of more anti-G MAbs (Table 4) with VHSV neutralizing activity [12, 13, 21] together with the sequence of the G protein [20] should help to identify the molecular characteristics of all these neutralization epitopes.

A paradoxical increase in viral replication at subneutralizing concentrations of antiviral Ab has been demonstrated for many families of viruses [4]. The concentration of anti-G MAb 1H10 at which neutralization ($> 300\,\mu g/ml$) or enhancement ($\leqslant 100\,\mu g/ml$) occurs, are of the same order of magnitude as those reported for other enhancements [4, 10]. However, the most studied type of enhancements is produced by anti-virus Abs which may or may not be neutralizing [5, 16] and which depend on the binding of Abs to the Fc and/or complement receptors present in the target cells [4, 17]. In contrast, the enhancement reported here seems to be unique for at least one neutralizing MAb. On the other hand, the binding of the anti-G MAb 1H10 to the G epitope might also require the presence of complement to produce neutralization, as demonstrated for the pike fry rhabdovirus with polyclonal Abs produced in rabbit [6]. No attempts (e.g., study the complement mediated neutralization) have been made to further investigate the underlying mechanism(s) but experiments are underway to study the possible in vitro enhancement by trout Abs.

In vivo enhancement has been observed both in natural situations [4] and under laboratory experimental conditions [11, 22] for other viruses. Thus it is possible that weak and host specific immune responses to some epitope(s) of rhabdoviruses (VHSV included) could enhance rather than reduce infectivity, and thereby worsen the course of the disease. In vivo evidence for this hypothesis has been presented in the rabies/rabbit model [18]. Also, some preliminary

Table 4. Characterization of the anti-VHSV G MAbs described to date

Name	Isotype	Neutralization		Binding			Reference
		in vitro	in vivo	M	G	E	
I	IgG_1	yes	yes	no	yes	?	[13]
II	IgG_1	no	yes	yes	yes	?	[13]
III	IgG_1	no	no	yes	yes	?	[13]
C10	IgG_{2a}	yes	?	no	yes	?	[15]
I10	IgG_1	no	?	yes	yes	?	[15]
1H10	IgG_1	yes	?	yes	?	yes	this work
1F10	IgG_{2b}	no	?	yes	?	no	this work

M Binding to protein G after β-mercaptoethanol treatment by immunoblotting
G Binding to protein G after deglycosylation
E Enhancement of VHSV infectivity at low concentration
? Test not published

evidence now exists with the VHSV/trout model, for instance, immunization by injection of trout with subunit G (obtained in bacteria and containing the epitope(s) defined by MAb 1H10) caused higher mortalities than control bacteria treated the same way (Thiry, pers. comm.). If so, this effect could have a major impact on defining vaccination strategies against the VHS disease. For instance, identification of the regions of the G-protein that are targets for the hypothetical enhancing trout Abs would need to be identified so that those regions are not included in a subunit vaccine. An antiviral vaccine should stimulate the production of Abs directed against functionally important epitopes and not against enhancing epitopes. Assuming that the mouse MAbs define similar epitopes as the trout Abs, parallel studies with the different neutralizing MAbs against VHSV obtained recently [13, 15] could help to identify the enhancing/neutralizing epitopes and to design proper subunit vaccines. On the contrary, if trout Abs react against other enhancing epitopes than those defined by the mouse MAbs, those different antigenic sites will need to be identified, for instance by fractionation of trout Abs using sequence specific G-peptides [20]. At present, at least one in vitro non-neutralizing anti-G MAb independent of disulphide bonding, can neutralize VHSV in vivo [13]. It is expected that these questions could be further clarified as the panel of available anti-G MAbs grows (Table 4). MAb 1H10 is unique in that it has some enhancement (first decribed here for VHSV) and neutralizing of infectivity yet it recognizes a fully denatured G protein contrary to other MAbs with neutralizing activity previously described (Table 4).

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