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Maternal cell-mediated immunity in normal and pre-eclamptic pregnancy

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SUMMARY

Maternal cell-mediated immunity (CMI) to fetal (paternal) HLA was studied in both normal and pre-eclamptic pregnancies. Maternal–paternal mixed lymphocyte reactivity (MLR) was assessed using macrophage migration inhibitory factor (MIF), leucocyte migration inhibitory factor (LIF) and blast transformation assays. Primary and secondary responses were differentiated by studying 6 day MLR time courses. Maternal lymphocytes (normal pregnancies) did not show an early, (i.e. secondary), proliferative response, nor did the pattern of LIF release suggest maternal CMI to fetal (paternal) HLA. However, the lymphocyte responses between pre-eclamptic couples, in terms of both proliferation and LIF release, were consistently and significantly different from those of normal couples; a finding which may reflect an abnormal immune response.

INTRODUCTION

Pre-eclampsia is a disorder of human pregnancy of unknown aetiology. Some features suggest that it may have an immune pathology (Redman, 1980): including the first pregnancy preponderance of the disorder (MacGillivray, 1958), the protective effect of previous abortions (MacGillivray, 1958) and an increased incidence of pre-eclampsia when a woman changes partners (Feeney, 1980). These observations could be explained if pre-eclampsia results from a relative failure of a specific maternal immunization to the conceptus which is essential for normal pregnancy (Redman, 1980). The secondary maternal immune response stimulated in later pregnancies by the same partner would then prevent its recurrence.

The mixed lymphocyte reaction (MLR) in normal human pregnancy, has been repeatedly examined with both maternal-paternal or maternal-fetal combinations but usually with respect only to the primary reaction developing at 5 days or later (Ceppellini *et al.*, 1971; Jenkins & Hancock, 1972; Jones & Curzen, 1973; Herva & Tiilikainen, 1977). These studies provide no information about prior maternal sensitization to the fetus, which can only be detected *in vitro* by demonstrating a secondary response—that is one which is earlier, and larger (Bondevik & Thorsby, 1974; Moen & Thorsby, 1978). The evidence for a secondary maternal response is conflicting. It has not been detected in time course studies of thymidine incorporation in MLR (Carr, Stites & Fudenburg, 1974; Herva & Jouppila, 1977; Moen *et al.*, 1980), but has been demonstrated in MLR by measuring the early production of macrophage migration inhibitory factor (MIF) (Rocklin *et al.*, 1973). Lymphokine measurements have also been used to demonstrate maternal CMI to

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placental antigens in both normal (Youtananukorn, Matangkasombut & Osathanondh, 1974; Stimson, Strachan & Shepherd, 1979) and pre-eclamptic pregnancies (Toder *et al.*, 1979).

We have therefore re-examined these issues in detail to determine if maternal cell-mediated immunity (CMI) to paternal HLA routinely develops in a normal first pregnancy, and if so whether it differs in pre-eclamptic women.

MATERIALS AND METHODS

Patients. Normal primigravid, normal multigravid (four or more pregnancies), and primigravid women with severe pre-eclampsia were studied. Pre-eclampsia was diagnosed by an increase in the systolic and diastolic arterial pressure of at least +30/+20 mmHg respectively, from the first reading taken in the first half of pregnancy; combined with terminal proteinuria (greater than or equal to 0·2 g/l); and regression of these symptoms after delivery. All the pre-eclamptic patients were primigravid and did not have blood transfusions. The normal patients had a normal pregnancy outcome with neither proteinuria nor a blood pressure of 140/90 or higher. Controls were women who had never been pregnant.

Separation of mononuclear cells. Maternal and paternal venous blood (60 ml) was collected into heparinized tubes (5 units/ml) 4–7 days post-partum. The mononuclear cells were separated on Ficoll-Paque (Pharmacia, Uppsala, Sweden), and resuspended at 10×10^6/ml in medium 199 (with Earles modified salts plus $NaHCO_3$, GIBCO, Paisley, Scotland) supplemented with 6·6 g/l HEPES, (Sigma, London), 50 units/ml antibiotic (penicillin and streptomycin) and 10% pooled heat-inactivated serum from healthy males (NHS). Mixed lymphocyte reactivity was determined by guinea-pig macrophage migration inhibition, leucocyte migration inhibition and blast transformation assays.

Guinea-pig macrophage migration assay. Mixed lymphocyte cultures were set up as described by Rocklin *et al.* (1973) with responder and stimulator cells in a ratio of 5:1. The stimulator cells were not inactivated. The culture medium contained RPMI 1640 (Flow, Irvine) supplemented with 50 units/ml antibiotics (penicillin and streptomycin) and 10% NHS. The cultures were incubated at 37°C in 5% CO_2 in air and the supernatants were collected after 24 hr and tested for MIF activity.

Migration of guinea-pig peritoneal exudate cells (PEC) was carried out by the capillary tube technique as previously described (Sargent & Salaman, 1980). The medium was RPMI 1640 as above with 15% guinea-pig serum. Test supernatants were diluted 1:3 in this medium.

The area of migration was projected and measured by planimetry. The mean of six replicates was calculated for each experimental group and results were expressed as migration indices:

$$\frac{\text{area of test MLR}}{\text{area of control}} \times 100$$

Controls were responder lymphocytes incubated alone. All measurements were read blind.

Human leucocyte migration assay. Mononuclear cells were diluted to $2{\cdot}5 \times 10^6$/ml in medium and 1 ml cultures were set up in 10 ml conical tubes (Sterilin, Teddington, England). Allogeneic mixtures contained responder and stimulator cells in a ratio of 1:1. Leucocyte inhibitory factor (LIF) production by stimulator cells was blocked by pre-incubation in medium 199 containing 500 μg/ml puromycin (Sigma, London) for 90 min and washing three times before addition to the cultures (Gorski *et al.*, 1976). Culture supernatants were collected each day for 6 days. The mononuclear cells were spun down at 400*g* for 10 min and the supernatants pipetted off and immediately frozen at −20°C.

All supernatants were assayed for LIF production within 2 weeks by the agarose well leucocyte migration technique of Clausen (1972) as modified by Bendtzen & Rocklin (1980). Polymorphonuclear indicator cells were obtained from volunteers at the Regional Blood Transfusion Centre, Oxford. Two diameters of each migration area from quadruplicate wells were measured using a microfilm reader fitted with a grid on the screen and the area calculated by the method of Weese, *et al.* (1978), excluding the area of the central well. Supernatants were randomised before testing and all measurements were carried out blind. The median areas for each group were calculated and results were expressed as migration indices as above.

Transformation assay. Mononuclear cells were diluted to 1×10^6/ml in medium 199 as above and triplicate 200 μl cultures were set up in flat-bottomed microtitre plates (Sterilin, Teddington). Allogeneic mixtures contained responder and stimulator cells in a ratio of 1 : 1. Stimulator cells were inactivated by irradiation (2,000 rad). Cultures were incubated at 37°C in 2% CO_2 in air. Blastogenesis was determined each day for 6 days. One half of a microcurie of ^{3}H-thymidine (TRK 120, 25 Ci/mmol, Amersham) was added to each well and after a further 18 hr incubation the cultures were harvested onto glass fibre filters using a Skatron harvester (Flow, Irvine) and the filters were counted by liquid scintillation counting. Results are expressed as the median counts per minute (c.p.m.) of the triplicate cultures.

Statistics. The differences within experimental groups were tested by the Wilcoxon test for pair differences, and differences between groups by the Wilcoxon rank sum test. Non-parametric tests were selected because the observations were not normally distributed.

RESULTS

MIF production in MLR

Twenty-six normal primigravid and seven multigravid women were studied 4–7 days post-partum. In the primigravid group there was no difference in the response of maternal to paternal lymphocytes (migration index $\pm$ s.e.m. $= 100{\cdot}2 \pm 4{\cdot}0$) compared to the reciprocal control culture (paternal to maternal lymphocytes $= 103{\cdot}2 \pm 3{\cdot}4$) (Wilcoxon test for pair differences). This was also true for the multigravid group where the maternal response was $87{\cdot}4 \pm 8{\cdot}7$ and the reciprocal control $90{\cdot}1 \pm 6{\cdot}8$. There was no significant difference between the responses of primigravid compared to multigravid women (Wilcoxon rank sum test).

MLR time course studies in normal pregnancy

Six day time course studies of MLR were investigated with parallel measurements of thymidine incorporation and LIF production. Eleven normal primigravid and 10 normal multigravid women were studied 4–7 days post-partum. Control cultures comprised cells from 10 nulligravid women and unrelated males. Technically satisfactory measures of LIF production were obtained in nine control, eight primigravid and eight multigravid experiments.

In control cultures the pattern of LIF production was that of a primary MLR, with a maximum response on days 4, 5 and 6 (Fig. 1a), (Clausen, 1972). A similar time course was seen with primigravid (Fig. 1b) and multigravid couples (Fig. 1c), although in the latter the response declined

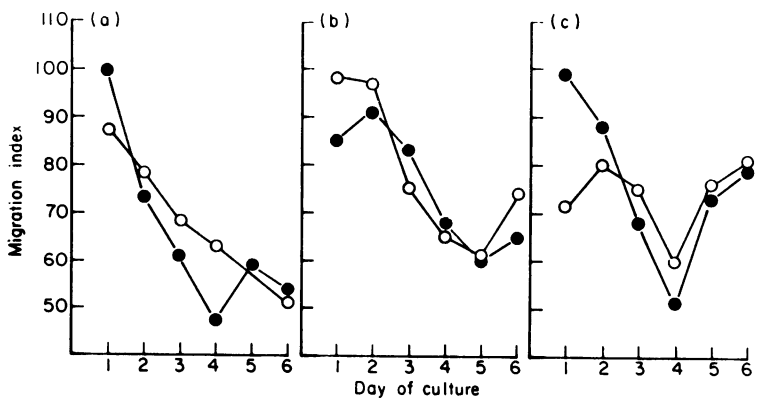

Fig. 1. Time course of LIF production in one way maternal (M) x paternal (P) MLR—normal pregnancy. The results are expressed as the median migration index of each experimental group; (●——●) $M_r \times P_s$, (○——○) $P_r \times M_s$. There were no significant differences between $M_r \times P_s$ combinations in MLR and reciprocal controls. (Wilcoxon test for pair difference). (r = responder cells; s = puromycin treated stimulator cells). (a) = control MLR; (b) = primigravid MLR; (c) = multigravid MLR.

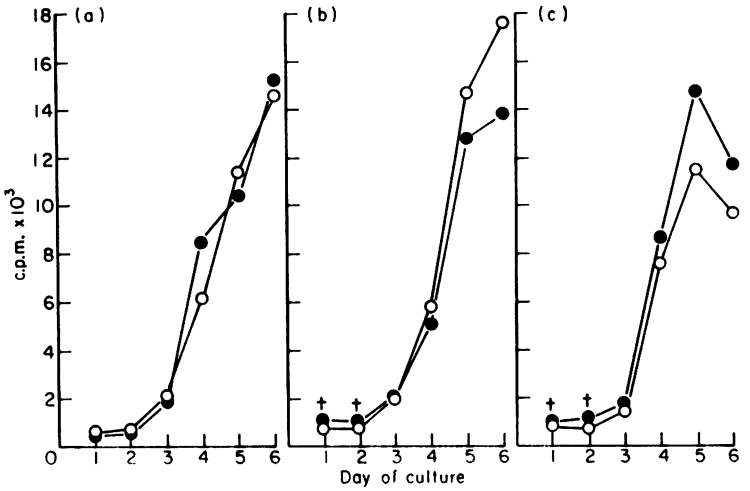

Fig. 2. Time course of lymphocyte proliferation in one way maternal (M) × paternal (P) MLR—normal pregnancy. Results are expressed as median c.p.m. of each experimental group. (●——●) $M_r \times \times P_s$. (○——○) $P_r \times M_s$. *$P < 0{\cdot}05$, †$P < 0{\cdot}01$ (Wilcoxon test for pair differences). [r = responder cells; s = irradiated stimulator cells]. (a), (b) & (c) same as for Fig. 1.

on days 5 and 6. The maternal cell responses did not differ significantly from the nulligravid female control responses nor from the paternal cell responses in reciprocal control cultures.

Blast transformation was assessed in parallel in the same cultures. The control and primigravid groups showed similar primary MLR time courses with a maximum response at 6 days (Fig. 2a, 2b). In the multigravid group the response paralleled that of LIF production, with a slightly earlier peak on day 5 and a small decline thereafter. Overall the responses were primary, not secondary in character.

These results demonstrated no evidence of maternal sensitization to paternal (fetal) HLA.

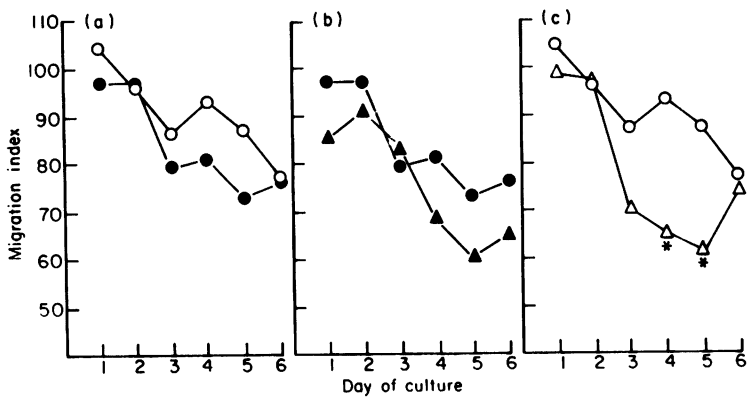

Fig. 3. Time course of LIF release in one way maternal (M) × paternal (P) MLR—pre-eclamptic pregnancy. Results are expressed as the median migration index of each experimental group. (●——●) pre-eclamptic $M_r \times P_s$, (○——○) pre-eclamptic $P_r \times M_s$, (▲——▲) normal primigravid $M_r \times P_s$, (△——△) normal primigravid $P_r \times M_s$. *$P < 0{\cdot}05$; Wilcoxon test for pair differences and rank sum test. [r = responder cells; s = puromycin treated stimulator cells]. (a) pre-eclamptic MLR; (b) pre-eclamptic + primigravid MLR $M_r \times P_s$, (c) pre-eclamptic + primigravid MLR $P_r \times M_s$.

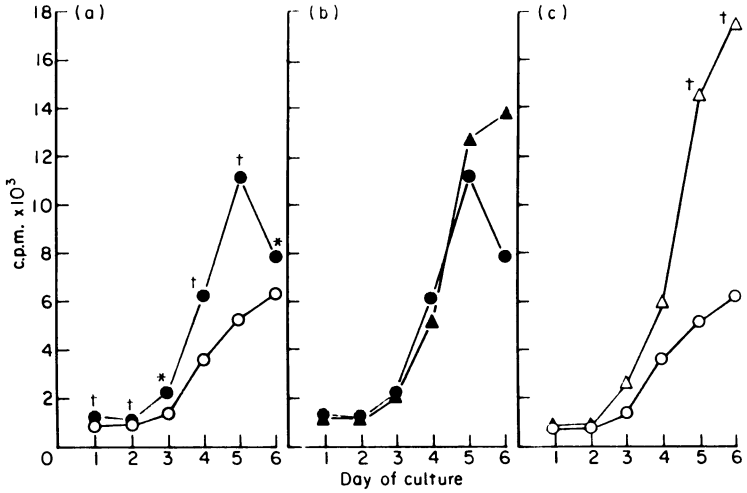

Fig. 4. Time course of lymphocyte proliferation in one way maternal (M) × paternal (P) MLR—pre-eclamptic pregnancy. Results are expressed as the median c.p.m. of each experimental group. (●——●) pre-eclamptic $M_r \times P_s$. (○——○) pre-eclamptic $P_r \times M_s$ (▲——▲) normal primigravid $M_r \times P_s$. (△——△) normal primigravid $P_r \times M_s$. $*P<0{\cdot}05$, $\dagger P<0{\cdot}01$; Wilcoxon test for pair differences and rank sum test. (r = responder cells; s = irradiated stimulator cells). (a), (b) and (c) same as for Fig. 3.

MLR time course studies in pre-eclamptic pregnancies

Thirteen primigravid women with pre-eclampsia and their husbands were studied 4–7 days after delivery. In nine studies technically satisfactory measures of LIF production were obtained. The maternal response was diminished compared to normal primigravid pregnancy (Fig 3a, 3b), but not significantly. However, on days 4 and 5 the pre-eclamptic paternal response was significantly less than the paternal response in the normal primigravid group (Fig. 3c).

Blast transformation was very different in the pre-eclamptic group (Fig. 4), with an asymmetry

Table 1. Time course of spontaneous lymphocyte transformation in post-partum women

	Lymphocyte donor	Day of culture 1	2	3	4	5	6
Non-pregnant controls	Male	678	604	524	521	518	454
	Female	777	479	368	428	539	725
Primigravid	P	818	759	750	840	820	1120
	M	1,632*	1,585*	894	790	811	825
Multigravid	P	795	841	485	600	516	616
	M	1,411*	1,573†	872†	528	464	410
Pre-eclamptic	P	632	470	513	503	468	886
	M	1,892†‡	1,445†‡	857*‡	547‡	524‡	753‡

The results are expressed as median c.p.m.

P = paternal, M = maternal, * = $P<0{\cdot}05$, † = $P<0{\cdot}01$.

P values refer to differences in thymidine uptake between maternal and paternal lymphocytes (Wilcoxon test for pair differences). ‡ = no significant difference between thymidine uptake in pre-eclamptic and either primigravid or multigravid women's lymphocytes (Wilcoxon rank sum test).

Table 2. Time course of one way mixed lymphocyte reactivity between a pre-eclamptic woman, her husband and her HLA-identical sister

HLA types						
Maternal	A1,3	B7,8	CW7	BW6		
Sister	A1,3	B7,8	CW7	BW6		
Paternal	A25,29	B18,44	—	BW4,6		
			Day of culture			
MLR combination	*1*	*2*	*3*	*4*	*5*	*6*
$P_r \times M_s$	817	533	1,768	6,382	4,681	4,434
$P_r \times SI_s$	814	687	2,965	12,813	13,880	8,802
$M_r \times P_s$	1,412	890	2,574	10,319	11,044	5,126
$SI_r \times P_s$	746	683	2,817	12,199	11,546	10,663
$M_r \times SI_s$	821	374	146	617	168	130
$SI_r \times M_s$	908	465	240	332	293	193

The results are expressed as median c.p.m. of triplicate cultures.
M = maternal, P = paternal, SI = sister.
[r = responder cells; s = irradiated stimulator cells].

between the maternal responses to paternal cells and paternal responses in the reciprocal control cultures. Throughout the time course maternal responses were significantly greater than the reciprocal paternal responses (Fig. 4a), but not greater in magnitude than those of normal primigravid women (Fig. 4b). Thus the asymmetry was caused by a significantly reduced paternal response (Fig. 4c). In summary, in terms of both LIF production and blast transformation, the paternal responses in MLR were significantly impaired.

The effect of spontaneous transformation on thymidine incorporation

The maternal responses were greater than paternal responses on days 1 and 2. The differences were small but consistent enough to be significant in all pregnant groups. These were shown to be due to higher spontaneous incorporation of ^{3}H-thymidine by pregnant compared to male cells alone; and not to specific maternal sensitization (Table 1). Furthermore, spontaneous transformation was similar in normal and pre-eclamptic primigravid women.

Studies of a pre-eclamptic woman and her HLA identical sister

The lack of paternal responsiveness in MLR in the pre-eclamptic group could be produced if the HLA phenotype of maternal lymphocytes were poorly stimulatory (e.g. homozygous at the D locus). To answer this question the responses of pre-eclamptic women and their HLA identical sisters are being studied. The results of the first experiments are shown in Table 2. The father's lymphocytes respond normally to the sister's lymphocytes but their response to the mother is suppressed. Thus it would appear that this suppression is not a consequence of the maternal MHC phenotype, and is a special feature of the disease.

DISCUSSION

Our data clearly demonstrate the absence of maternal cellular sensitization to paternal HLA. This is true for both MIF and LIF production in the MLR as well as blast transformation. In the latter two instances our conclusion rests on time course studies which show in detail the primary nature of the maternal lymphocyte response. Even in women of high parity (four or more pregnancies) we could not demonstrate specific maternal cell-mediated immunity.

These observations confirm the results of earlier time course studies of maternal blast transformation responses in MLR either to fetal (cord blood) cells (Carr *et al.*, 1974; Moen *et al.*, 1980) and paternal cells (Herva & Jouppila, 1977; Moen *et al.*, 1980). Similar investigations using LIF production as an index of sensitization have not been reported before.

Some reports show a decreased maternal proliferative response to paternal lymphocytes compared with that against unrelated cells in one way (Ceppellini *et al.*, 1973) or two way MLR (Jenkins & Hancock, 1972). Similarly, depressed maternal responses to fetal (cord blood) lymphocytes have been reported (Carr *et al.*, 1974; Herva & Tiilikainen, 1977). The latter may be discounted because the observations were not controlled for the haplotype identity between mother and child. We find no evidence for suppression in mother–father combinations in agreement with several other investigators (Carr *et al.*, 1974; Moen *et al.*, 1980).

Overall, the *in vitro* evidence for maternal sensitization to fetal (paternal) HLA rests solely on the work of one group measuring MIF production in MLR (Rocklin *et al.*, 1973, 1976). We are unable to confirm their results either directly or indirectly.

Other claims for *in vitro* evidence of maternal sensitization to the fetus are based on studies with trophoblast antigens stimulating either MIF (Youtananukorn *et al.*, 1974) or LIF production (Stimson *et al.*, 1979). The syncytial membranes of trophoblast do not exhibit MHC antigens (Sunderland *et al.*, 1981); nor are they capable of stimulating blast transformation in maternal lymphocyte culture. However, if the maternal cellular reaction to these trophoblast preparations were to fetal HLA from contaminating non-trophoblast cells then this would have been mirrored in MLR with either fetal or paternal lymphocytes.

The lack of HLA on villous trophoblast may explain how the fetus avoids maternal immune rejection, but not the lack of materno–fetal CMI which we have observed. The common (and clinically normal) development of maternal alloantibodies during gestation (Oberweg & Engelfreit, 1969) indicates maternal contact with, and immune responses to, fetal HLA. Early human non-villous trophoblast, in contrast to villous trophoblast, exhibits HLA-A,B,C, (but not HLA-D_R) (Sunderland, Redman & Stirrat, 1981) and it may be at this level that maternal exposure occurs.

We conclude that *in vivo* there must be a block of either the development or expression of cell-mediated sensitivity to fetal HLA. Maternal lymphocytes can be primed in the normal way to fetal lymphocytes *in vitro* (Moen *et al.*, 1980), therefore any block must be easily removed and is unlikely to be the result of suppressor cell activity. Pregnancy serum contains a factor (probably IgG) which blocks cell-mediated responses *in vitro* (Bissenden, Ling & MacKintosh, 1980). Our results suggest that a putative blocking factor would act on the afferent arm of the immune response, preventing maternal sensitization rather than by blocking the activity of sensitized lymphocytes as suggested by Rocklin (1976). Furthermore, Rocklin has only found sensitization in some (mainly multigravid) women, and it is probable that this occurs at delivery as in Rhesus disease and is not a part of a normal first pregnancy.

Studies of maternal lymphocye function in pre-eclampsia are limited. Spontaneous lymphocyte transformation has been shown to increase during allograft rejection (Page *et al.*, 1971) and it has been suggested that this may also occur in pre-eclampsia. Our results confirm those of Gaugas, Jones & Curzen (1975) and Petrucco *et al.* (1976) showing an increase in spontaneous transformation in all pregnant women, but with no evidence of hyperreactivity in pre-eclampsia.

MLR time courses in pre-eclampsia have not been studied before. Our results show a consistent and significant difference between maternal and paternal responses, due to a diminished paternal, rather than an enhanced or secondary maternal reaction. No other studies are comparable because of differences in technique and design and provide no firm data to support or refute our observations (Jenkins *et al.*, 1978; Halbrecht & Komlos, 1974; Gille, Williams & Hoffman, 1977; Curzen, Jones & Gaugas, 1977).

It is unlikely that the depressed paternal cell response is the result of a poor stimulatory capacity of the mother's lymphocytes as the father is able to give a normal MLR with an HLA identical sister. Another possibility is that primed maternal cytotoxic cells are present, which could suppress the MLR by eliminating paternal responder lymphocytes, although these would need to be resistant

to irradiation. Alternatively, suppressor cells may be involved. Whatever its cause, this suppression is a feature of pre-eclamptic pregnancy. Its nature and specificity is being investigated further.

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