Immunology 1989 **67** 435–440

Presentation of myelin basic protein by normal guinea-pig brain endothelial cells and its relevance to experimental allergic encephalomyelitis

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Accepted for publication 3 May 1989

SUMMARY

Previous studies have shown that endothelial cells in the central nervous system (CNS) of normal guinea-pigs constitutively express certain MHC class II determinants, whilst the expression of other determinants is apparent during the acute phase of chronic relapsing experimental allergic encephalomyelitis (CREAE). The expression of MHC class II determinants is retained by endothelial cells derived from normal guinea-pig brain tissue and maintained in culture. This present study demonstrates that the MHC class II molecules on these cells can be recognized by allogeneic lymphocytes, resulting in a proliferative response which is enhanced by the addition of exogenous IL-2. The endothelial cells were incapable of presenting either purified protein derivative or ovalbumin, but they could present autologous myelin basic protein (MBP), an encephalitogen implicated in the pathogenesis of EAE. The resulting lymphocyte proliferative response was of the same magnitude as that obtained when a control population of macrophages was used to present MBP. These results, therefore, suggest that cerebrovascular endothelia have the potential to play a role in the pathogenesis of EAE.

INTRODUCTION

The expression of major histocompatibility complex (MHC) class II antigens by an accessory cell is a prerequisite for effective antigen presentation to T-helper lymphocytes (Rosenthal & Shevach, 1973). A number of cell types, besides the classical macrophage, have been shown to express MHC class II antigens and to present exogenous antigen to appropriately sensitized syngeneic lymphocytes in *in vitro* antigen presentation assays. These include B lymphocytes (Chesnut, Colon & Grey, 1982), epidermal Langerhans' cells (Stingl *et al.*, 1978), dendritic cells of lymphoid organs (Gautam & Glynn, 1989), and keratinocytes which have been induced to express MHC class II antigens (Gaspari & Katz 1988).

Endothelial cells are situated in a prime position to participate in cell-mediated immune reactions, particularly in central nervous system (CNS) disorders where they are the predominant cell type which constitute the blood–brain barrier (BBB). Several studies have shown that in 'normal' human and animal CNS tissue, vascular endothelial cells do not express MHC class II antigens, but that expression of these antigens is apparent on endothelia during the active phases of multiple sclerosis (MS) (Traugott, Scheinberg & Raine, 1985b) and experimental allergic encephalomyelitis (EAE) (Antoniou *et al.*, 1987; Sobel *et al.*, 1984). Recent studies have shown that CNS endothelia, in normal Strain 13 guinea-pig tissue, constitutively express certain MHC class II determinants, whilst other determinants become detectable during the course of chronic relapsing EAE (CREAE) (Butter *et al.*, 1988; Wilcox *et al.*, 1989). The constitutive expression of MHC class II antigens is retained when endothelial cells, derived from normal CNS tissue, are propagated in tissue culture (Wilcox *et al.*, 1989). The results of these studies suggest that CNS endothelia may have the potential to act as local antigen-presenting cells at the site of the BBB and contribute to the pathogenesis of CREAE.

To elucidate this further, brain-derived endothelia from Strain 13 guinea-pigs were tested for their ability to act as stimulators of allogeneic T-lymphocyte proliferation and as accessory cells in the presentation of purified protein derivative (PPD), ovalbumin (OVA) and myelin basic protein (MBP) to T lymphocytes. The results suggest that CNS endothelia could play a role in guinea-pig CREAE as they were able to present MBP to previously sensitized T lymphocytes.

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MATERIALS AND METHODS

Animals

Inbred Strain 13 guinea-pigs (University of York, York) and

Strain 2 guinea-pigs (Royal College of Surgeons, London) were used. These strains have been shown to differ genetically only at their MHC class II loci (Schwartz, Paul & Shevach, 1976).

Sensitization protocols

Animals were injected at between 18 and 21 days of age as follows.

Sensitization of Strain 2 guinea-pigs to alloantigen. Strain 13 guinea-pigs were injected intraperitoneally with 15 ml sterile paraffin oil. Four days later the peritoneal exudate cells (PEC) were collected and washed twice in RPMI-1640 medium (Gibco, Paisley, Renfrewshire). 0·05 ml of medium containing 5×10^7 PEC was injected subcutaneously into the dorsum of each hind foot of Strain 2 guinea-pigs. The draining popliteal and inguinal lymph nodes were removed 9 days later and served as the source of Strain 13 MHC class II-sensitized lymphocytes.

Sensitization of Strain 13 guinea-pigs to the antigens PPD, OVA, and MBP. Guinea pigs were sensitized to PPD, OVA and MBP by injecting 0·1 ml of an antigen/adjuvant homogenate into the dorsum of each hind foot. The inoculums consisted of either: (i) PPD [1 ml PBS; 1 ml incomplete Freund's Adjuvant (IFA); 10 mg *Mycobacterium tuberculosis* $H_{37}Ra$ (Difco Lab, East Molesey, Surrey)]; (ii) OVA 1 ml PBS; 1 ml complete Freund's Adjuvant (Difco); 100 μg chicken ovalbumin (Sigma Chemical Company, Poole, Dorset); or (iii) MBP (1 ml PBS; 1 ml IFA; 10 mg *M tuberculosis* $H_{37}Ra$; 1 mg guinea-pig MBP (kindly provided by Dr P. Glynn, Multiple Sclerosis Laboratory, Institute of Neurology, London. The animals were killed 11 days post-inoculation and the inguinal and popliteal lymph nodes removed.

Preparation of T lymphocytes

The draining lymph nodes from the sensitized guinea-pigs or the mesenteric lymph nodes from normal animals were removed, trimmed of excess tissue and teased through a steel gauze. A suspension of T lymphocytes was obtained by purification on a nylon-wool column (Leukopak Leucocyte Filter, Fenwal Laboratories, Division of Travenol Laboratories Incorporated, Deerfield, IL) followed by incubation of the nylon-wool non-adherent cells in plastic petri-dishes for 90 min at 37°, to remove any remaining adherent cells from this population. The non-adherent cells were washed off and resuspended at a final concentration of 6×10^6 cells per ml in the complete medium which consisted of RPMI-1640 with 10% fetal calf serum, 4 mM glutamine, 400 U/ml penicillin, 400 μg/ml streptomycin and 2 mM sodium pyruvate (Flow Laboratories, Rickmansworth Ltd, Herts).

This population consisted of between 95% and 97% T lymphocytes, and 3% and 5% B lymphocytes, as assessed using the mouse anti-guinea-pig pan T and pan B-lymphocyte monoclonal antibodies, MSgp7 and MSgp9 (Dept. of Pathology, Royal College of Surgeons of England) (C. Butter, D. G. Healey, D. Baker and J. L. Turk, manuscript submitted for publication; Healey, Agha & Turk, 1988).

Preparation of accessory cell populations

Cerebral endothelial cells. The blood vessels were isolated from the brains of normal adult Strain 13 guinea-pigs as previously described (Wilcox *et al.*, 1989). The digested vessels were maintained in a 250 ml Lux tissue culture flask (Flow Laboratories Ltd) in medium 199 containing 30% fetal calf serum, 4 mM glutamine and 400 U/ml penicillin and 400 μg/ml streptomycin, at 37°, until the endothelial cells had migrated out of the vessel, divided, and covered 60–70% of the plastic. The endothelia were then removed by trypsin digestion, using 0·1% trypsin solution dissolved in medium 199. The cells were washed twice in RPMI-1640 and plated out into 96-well flat-bottomed Nunc tissue culture plates (Gibco) at concentrations of 5×10^3, 1×10^4, 2×10^4 and 5×10^4 cells per well. The cells were left to adhere overnight at 37° in RPMI-1640 containing 30% fetal calf serum, glutamine and penicillin/streptomycin. After this culture the medium was replaced with complete medium and the cells were irradiated with 2000 rads.

The purity of the endothelial cell population was assessed by subculturing some of the cells onto plastic coverslips (Thermanox Plastic Coverslips, Flow Laboratories). After overnight culture the cells were washed in RPMI-1640 medium and fixed in acetone for 10 min. The antibodies to factor VIII-related antigen and angiotensin-converting enzyme (ACE) were used and stained greater than 95% of the cells. In addition, antibodies to desmin (Dako Ltd, High Wycombe, Bucks) indicated the presence of 3–5% contaminating smooth muscle cells. Antibody to glial fibrillary acid protein (GFAP) (kindly provided by Dr N. Woodroofe, Multiple Sclerosis Laboratory, London) and non-specific esterase staining failed to show the presence of contaminating astrocytes and macrophages, respectively.

PEC. Oil-induced PEC were prepared as described previously. The cells were plated out at 5×10^3, 1×10^4, 5×10^4, 1×10^5 and 5×10^5 cells per well in 100 μl of complete medium. They were irradiated with 1800 rads.

Allogeneic lymphocyte proliferation assay

3×10^5 normal or Strain 13 PEC-sensitized Strain 2 lymphocytes were incubated with either Strain 13 endothelia or PEC in the presence or absence of 100 U/ml recombinant human interleukin-2 (IL-2) (Hoffman La Roche, Nutley, NJ). The cells were incubated together at 37°, for 4 and 6 days. Tritiated thymidine ([^{3}H]TdR, Amersham International, Bucks), at a final concentration of 1 μCi per well, was added to the cultures 18 hr prior to harvesting. The cells were MASH harvested and the incorporation of [^{3}H]TdR assessed using a liquid scintillation counter.

Antigen-presentation assays

3×10^5 appropriately sensitized T lymphocytes were incubated for 72 hr with the CNS endothelia or PEC in the presence or absence of antigen. PPD was used at a final concentration of 25 μg/ml, OVA at 100 μg/ml and MBP at 50 μg/ml. Normal Strain 13 mesenteric T lymphocytes were cultured with accessory cells and 5 μg/ml concanavalin A (Con A; Pharmacia, Uppsala, Sweden). [^{3}H]TdR was added to the cultures 18 hr prior to harvesting.

Statistical analysis

The results are expressed as mean $\pm$ SD of at least three to four wells per experimental group. The significant differences between groups were assessed using the Student's *t*-test with appropriate modification where the *F*-test showed significant sample variances. Each experiment was repeated at least three times.

Table 1. Proliferative response of sensitized Strain 2 lymphocytes cultured with Strain 13 brain-derived endothelial cells. Results expressed as c.p.m. [^{3}H]TdR uptake

Accessory cell number	Proliferation after 4 days			Proliferation after 6 days		
	No T cells	With T cells	With T cells and IL-2	No T cells	With T cells	With T cells and IL-2
5×10^3	691±126	1147±318	3854±351*	386±110	1433±627	12,242±2555†
1×10^4	1264±317	2850±443‡	7258±1874†	575±63	5615±1125‡	16,093±945‡
2×10^4	1733±1070	3774±1101	8619±911‡	828±473	8714±930*	16,841±783*
5×10^4	—	—	—	750±167	4567±258‡	7542±1426†
None	N/A	65±22	253±46	N/A	87±53	70±29
1×10^4 PEC control	68±40	18478±2997	24236±3025	77±28	40904±4669	97117±8789
1×10^5 PEC control	96±67	80,809±15788	154448±2656	84±26	30200±303	58961±13420

*$= P < 0.001$; †$= P < 0.02$; ‡$= P < 0.01$ compared with the sum of counts for the endothelia and T cells cultured separately.

Table 2. Proliferative response of normal Strain 2 lymphocytes cultured with Strain 13 PEC. Results expressed as c.p.m. [^{3}H]TdR uptake

Accessory cell number	Proliferation after 4 days			Proliferation after 6 days		
	No T cells	With T cells	With T cells and IL-2	No T cells	With T cells	With T cells and IL-2
5×10^3	186±104	500±62	1602±184	109±16	131±22	2375±6522
1×10^4	73±30	1120±357	3968±406	19±5	752±177	14023±3359
2×10^4	278±201	2497±69	6727±1307	52±5	2489±1315	24074±2159
5×10^4	36±8	8070±845	15282±1431	45±6	18537±3749	116321±24283
1×10^5	294±319	16657±2292	32638±4201	212±145	36082±7405	141376±16663
5×10^5	105±22	5478±1927	14484±9116	75±14	1007±109	14882±3564
None	N/A	228±189	558±165	N/A	162±49	183±61

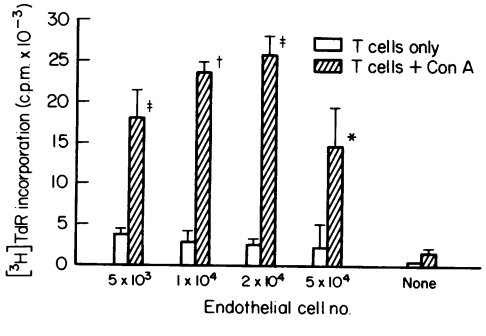

Figure 1. Proliferative response obtained when 3×10^5 normal Strain 13 T lymphocytes were cultured with Strain 13 endothelial cells and 5 μg/ml Con A. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$ compared with groups with accessory cells and T cells only.

RESULTS

Alloantigenic T-cell proliferation assays

Brain-derived endothelial cells from Strain 13 guinea-pigs were used as the stimulating cell population in a primary and secondary allogeneic reaction to determine whether or not lymphocytes can recognize the MHC Class II molecules previously detected on the endothelial cell surface. When T cells, derived from normal Strain 2 guinea-pigs, were cultured with the endothelial cells, no response was detected after either the 4-day or the 6-day culture period (data not shown). When lymphocytes, taken from Strain 2 animals which had previously been inoculated with Strain 13 PEC, were cultured with the endothelial cells, there was evidence of proliferation after 4 days in culture, particularly in the groups where exogenous IL-2 had been added. After 6 days in the culture the response was more pronounced, even in the absence of IL-2 (Table 1).

The control experiments showed that PEC were able to stimulate a primary allogeneic reaction even in the absence of

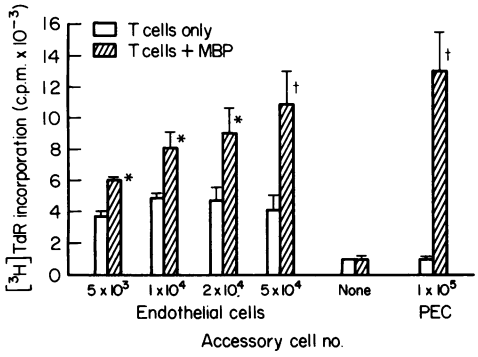

Figure 2. The presentation of MBP to syngeneic lymphocytes by brain-derived Strain 13 endothelial cells; 3×10^5 sensitized T lymphocytes were cultured with various numbers of endothelial cells or 1×10^5 oil-induced PEC, and 50μg/ml MBP. * $P < 0{\cdot}01$; † $P < 0{\cdot}001$ compared with groups with accessory cells and T cells only.

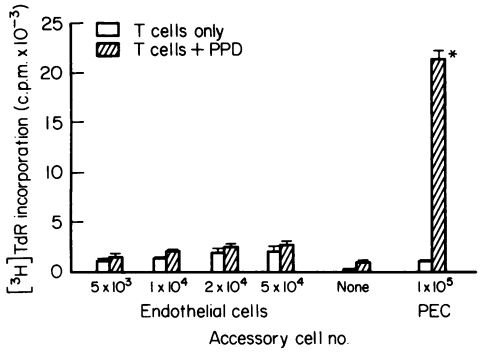

Figure 3. The presentation of PPD to lymphocytes; 3×10^5 sensitized Strain 13 T lymphocytes were cultured with various numbers of Strain 13 endothelial cells or 1×10^5 PEC, and 25 μg/ml PPD. * $P < 0{\cdot}001$ compared with groups with accessory cells and T cells only.

IL-2; after only 4 days in culture there was evidence of a T-cell response, but proliferation was greater after 6 days (Table 2). The lymphocytes taken from previously sensitized Strain 2 guinea-pigs showed dramatic proliferation in response to the PEC even after 4 days in culture. After 6 days fewer stimulator cells were necessary to induce maximum proliferation (Table 1).

Antigen- and mitogen-induced T-cell proliferation assays

Having established that the MHC class II molecules present on the Strain 13 endothelial cell can be recognized by lymphocytes, these endothelial cells were used as the accessory cell population in antigen- and mitogen-induced proliferation of syngeneic T cells. Con A was used as the T-cell mitogen and PPD, OVA and MBP were the antigens used in these assays.

Figure 1 shows a representative set of results obtained when endothelial cells were used to support Con A-induced T-cell proliferation. In this particular experiment the optimum response induced by the PEC control was 45,515 $\pm$ 6964 c.p.m., approximately twice the response obtained with the endothelial cells acting as the accessory cell population. PEC consistently induced a higher proliferative response, ranging between 1·5 and five times greater.

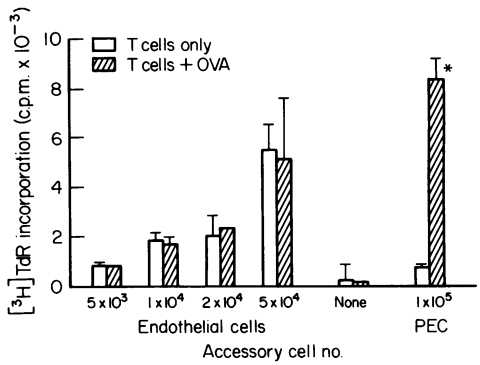

Figure 4. The presentation of OVA to lymphocytes; 3×10^5 sensitized Strain 13 T lymphocytes were cultured with Strain 13 endothelial cells or 1×10^5 PEC, and 100 μg/ml OVA. * $P < 0{\cdot}001$ compared with groups with accessory cells and T cells only.

When the endothelial cells were used to present antigen, it was found that they could present MBP to appropriately sensitized lymphocytes, but not PPD or OVA. Figure 2 depicts a typical set of data obtained when MBP was the antigen used. It can be seen that the extent of the response is dependent upon the number of endothelial cells present in the reaction and that the degree of proliferation is comparable to that obtained when PEC were used as the antigen-presenting cell type. Endothelial cells consistently failed to present either PPD or OVA and Figs 3 and 4 show a representative set of data obtained in these experiments. It should be noted that the endothelial cells, although irradiated, still take up a relatively high amount of [^{3}H]TdR as compared with the control PEC population, the amount incorporated depending upon the number of endothelial cells present (data not shown). This uptake of [^{3}H]TdR by endothelial cells accounts for the increasingly high levels of [^{3}H]TdR incorporation seen, particularly in Fig. 4, when T cells only were cultured with the varying concentrations of endothelial cells. The PEC were capable of presenting all three antigens (see control group of Figs 2, 3 and 4). With all three antigens some presentation by the PEC was evident, using as few as 5×10^3 cells per well, with 1×10^5 cells per well being the appropriate cell number to produce an optimum response.

DISCUSSION

Brain-derived endothelial cells, which have previously been shown to express certain MHC class II determinants, were used as the accessory cell population in *in vitro* lymphocyte proliferation assays. Experiments were designed to determine whether this cell type could present alloantigen, support a mitogen-induced T-cell proliferation response and present the antigens PPD, OVA and MBP to previously sensitized syngeneic lymphocytes. In all three studies the immunological functions of the endothelial cells were compared to the classical macrophage, present in oil-induced peritoneal exudates.

Trypsinization of the primary endothelial cell cultures results in single cells as well as small clumps of cells which can not be completely disaggregated before plating out; therefore, the actual number of cells being added to each well is not constant. In addition, the number of cells which adhere to the plastic during the overnight period can not be ascertained. However, by making an attempt to titrate the cells out at the

four approximate concentrations described, a cross-section of cell numbers can be produced and, therefore, the possibilities of either using too few accessory cells or saturating the system with too many accessory cells could be eliminated. This latter phenomenon is particularly evident when 5×10^5 PEC per well are used to stimulate an allogeneic reaction as opposed to 1×10^5 PEC per well, as can be seen in Table 2.

In the alloantigen-induced T-cell proliferation assays it was found that the endothelial cells were unable to instigate a primary allogeneic response, whereas they could stimulate the proliferation of allogeneic lymphocytes previously sensitized to the Strain 13 MHC class II molecules, particularly in the presence of exogenous IL-2. The PEC, in comparison, were potent alloantigen-presenting cell in both a primary and secondary response. It is unlikely that the proliferation observed in the secondary response was a result of an endothelial cell-specific stimulating antigen, as proliferation to this would have occurred in the primary assay. As no other macrophage or other antigen-presenting cells were present in these endothelial cell cultures, it would appear that the MHC class II molecules on the endothelial cell surface can be recognized by allogeneic lymphocytes, resulting in proliferation. The addition of IL-2 does appear to be necessary to achieve a greater responsiveness. The inability of the endothelia to induce a primary response may be the consequence of low levels of alloantigen expression on the cell surface. Guinea-pig brain-derived endothelial cells have been shown to express the determinants recognized by the monoclonal reagents MSgp8, 27E7 and HLA-DR (Wilcox *et al.*, 1989), which have been shown to react with the common determinants shared by both Strain 2 and Stain 13 guinea-pigs (Burger *et al.*, 1981; Healey *et al.*, 1988). The strain 13-specific determinant, recognized by the antibody CI.13.1, was not detectable by immunostaining on the endothelia (Wilcox *et al.*, 1989). Therefore, it is possible that these cells do not possess sufficient levels of alloantigen to stimulate a primary reaction. The primed lymphocytes, however, may either respond to lower levels of alloantigen or, being activated, produce cytokines with interferon-gamma-like activity, which induce additional alloantigen expression. An induction of such determinants would account for the peak proliferative response at Day 6 of the culture period.

The brain-derived endothelial cells were also tested for their ability to present antigen to sensitized lymphocytes. Although different concentrations of antigens were used to induce sensitized T lymphocytes, these cells generally produced similar proliferative responses when stimulated by PEC (data not shown) with all three of the antigens used in this study. However, the endothelial cells were capable of presenting only MBP. This ability to present MBP is consistent with previous reports which have shown that murine cerebrovascular endothelia, having been induced to express MHC class II antigens, can also present xenogeneic MBP to primed lymphocytes (McCarron *et al.*, 1986). In the majority of experiments performed the endothelia were as efficient as the PEC in the presentation of this particular antigen. However, experiments using rat brain-derived endothelial cells have shown that a weak lympho-proliferative response can be induced when these cells were used as the accessory cell population in the presentation of OVA to appropriately sensitized lymphocytes, when indomethacin was present (Pryce, Male & Sedgwick, 1989).

OVA, as well as other antigens such as keyhole limpet haemocyanin and hen egg lysozyme, has previously been shown to require processing before it can be successfully presented to T lymphocytes (Babbitt *et al.*, 1985; Chain, Kay & Feldmann, 1986; Shimonkevitz *et al.*, 1983; Streicher *et al.*, 1984). Consequently, one possible explanation for the inability of the endothelial cells to present OVA and PPD is that they can not process the antigen into a suitable configuration for the relevant antigenic epitopes to be presented in association with the MHC class II molecule. Cerebral vascular endothelia have been shown ultrastructurally to possess few phagocytic and pinocytic vesicles. Therefore, the cells' inability to present certain antigens may result from a failure to internalize the antigen in preparation for intracellular degradation. One recent report suggests rat dendritic cells could present MBP to lymphocytes, even in the presence of the lysosomotropic agent chloroquine (Gautam & Glynn, 1989). In similar studies, the dendritic cells were shown to be incapable of presenting OVA (P. Glynn, personal communication).

Thus, this study demonstrates that normal guinea-pig endothelia have the ability to present alloantigen and antigen. This is similar to other studies where non-classical antigen-presenting cells, such as keratinocytes, have been induced to express MHC class II antigens. It has been shown that keratinocytes present alloantigen in a secondary, but not a primary, reaction and present processed, but not native, antigen (Gaspari & Katz, 1988). However, this study demonstrates that guinea-pig brain endothelia can present autologous MBP, an encephalitogen implicated in the pathogenesis of EAE. This finding, together with the observations that certain MHC class II determinants are differentially expressed during the active stages of EAE (Antoniou *et al.*, 1987; Sobel *et al.*, 1984; Traugott, Raine & McFarlin, 1985a), and that MBP has been demonstrated on the surface of endothelial cells *in situ* (Traugott *et al.*, 1985a), suggest that the cerebrovascular endothelial cell has the potential to play a role in the pathogenesis of EAE and, by implication, MS.

ACKNOWLEDGMENTS

The authors would like to thank Miss J. O'Neill and Mr P. Papasavva for their expert technical assistance, Dr D. Parker and Mr C. Butter for their help and advice and Mrs J. Saxby for typing this manuscript.

The Multiple Sclerosis Society of Great Britain and Northern Ireland is gratefully acknowledged for its financial support.

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