Inhibition of the binding of $^{125}$I-labelled epidermal growth factor to mouse cells by a mitogen in goat mammary secretions

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Pre-colostrum and colostrum from goats cause a marked inhibition of the binding of $^{125}$I-labelled epidermal growth factor ($^{125}$I-EGF) to Swiss 3T3 cells. The ability of these secretions to inhibit $^{125}$I-EGF binding is closely correlated with the ability to stimulate DNA synthesis in quiescent 3T3 cell cultures, suggesting that goat mammary secretions may contain an EGF-related mitogen. However, the material in colostrum which inhibits $^{125}$I-EGF binding to Swiss 3T3 cells is a basic protein with $M_r > 20000$ and is thus quite different from mouse and human EGF. Furthermore, the colostral-mediated inhibition of $^{125}$I-EGF binding, although rapid and apparently competitive, differs from the inhibition of binding induced by native, unlabelled EGF. Thus, the inhibitory effect of colostrum is markedly decreased when the assay temperature is shifted from 37°C to 4°C whereas unlabelled EGF is an effective competitive inhibitor at both 37°C and 4°C. Incubation of cells with EGF causes a reduction in cell surface EGF receptors whereas exposure to colostrum does not induce down-regulation of the EGF receptor. Our results suggest that the colostral factor does not bind directly to EGF receptors but inhibits $^{125}$I-EGF binding by an indirect mechanism which involves a temperature-sensitive step.

EGF was first isolated from mouse submaxillary gland (Cohen, 1962) and a closely related polypeptide was subsequently purified from human urine (Cohen & Carpenter, 1975; Gregory, 1975). Both mouse and human EGF are potent stimulators of DNA synthesis and cell proliferation in a wide variety of cell types in culture (Gospodarowicz et al., 1978; Carpenter, 1981).

Colostrum and pre-colostrum from goats, sheep and cows contain material which inhibits the binding of $^{125}$I-EGF to its cellular receptors on mouse 3T3 fibroblasts (Brown & Blakeley, 1982). The ability of these secretions to inhibit $^{125}$I-EGF binding was closely correlated with their ability to stimulate DNA synthesis and proliferation in these cells (Brown & Blakeley, 1982). EGF is known to be present in mouse and human milk (Hirata & Orth, 1979a,b) and our results suggest that an EGF-like factor may also be present in the mammary secretions of various ungulates. We have therefore investigated the characteristics of the inhibition of $^{125}$I-EGF binding by colostrum and the biochemical properties of the inhibitory material in more detail. The results indicate that colostrum contains a polypeptide growth factor which reduces the affinity of the EGF receptor for EGF. However, the factor differs biochemically from mouse and human EGF and the mechanism by which the factor inhibits EGF binding to its receptor is apparently indirect.

Materials and methods

Materials

Culture medium, antibiotics, trypsin and newborn calf serum were from Flow Laboratories. Na$^{125}$I and [3H]thymidine were from Amersham International. EGF was prepared from male mouse submaxillary glands by the method of Savage & Cohen (1972). The lactoperoxidase procedure (Thorell & Johansson, 1971) was used to prepare $^{125}$I-EGF. The labelled protein was separated from unreacted Na$^{125}$I by passage of the iodination mixture through a Sephadex G-25 column equilibrated and eluted with a buffer containing 0.01M-phosphate, pH 7.4, and 0.15M-NaCl. The labelled EGF was stored frozen in the presence of 0.1% bovine serum albumin. The specific activity of the $^{125}$I-EGF, at preparation, was 85000–135000 c.p.m./ng. Antiserum to EGF was raised in rabbits as described (Brown & Holley, 1979). The
IgG fraction from the antiserum was prepared by the method of Fahey (1967) and linked to CnBr-activated Sepharose (Pharmacia) following the manufacturer's instructions.

Cell cultures

Stock cultures of Swiss mouse 3T3 cells were maintained on 9 cm plastic culture dishes (Nunc) and passaged every three days in DME medium containing 10% (v/v) newborn calf serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Dishes were incubated at 37°C in a humidified atmosphere of CO₂/air (1:9). For experimental use cells were seeded into 3 cm plastic dishes (Nunc) or 24-well cluster trays (Linbro) and grown for at least 6 days. These cultures were 'quiescent' as judged by a low level of [³H]thymidine incorporation. After a continuous 30 h exposure to [³H]thymidine, less than 1% labelling of cell nuclei could be detected by autoradiography (Brown & Holley, 1979).

Cell counting

Cells were rinsed with 1–2 ml of Ca²⁺- and Mg²⁺-free phosphate-buffered saline which contained 0.43 mM-EDTA and 0.05% trypsin. Samples (2 ml) of this solution were then added per 3 cm dish. After 5 min incubation at 37°C the cultures could readily be dispersed into a suspension of single cells by gentle pipetting. An aliquot of this suspension was diluted into Isoton and cells were counted on a Coulter Counter (model ZB1).

Collection and preparation of mammary secretions

All samples of pre-colostrum (pre-partum secretions), colostrum (0–24 h post-partum), and milk (later than 24 h post-partum) were collected by hand milking. The pre-partum samples were kept to a minimal volume in order to avoid initiating lactogenesis (Linzell & Peaker, 1974). The samples were centrifuged for 30 min at 30000 g and the aqueous phase withdrawn from below the floating lipid. The casein from some samples was precipitated by lowering the pH to 4.3 by the dropwise addition of 1 M-acetic acid.

¹²⁵I-EGF binding assay

Confluent cultures of cells were washed with 1–2 ml of binding medium which consisted of DME containing 0.1% crystalline bovine serum albumin, 0.1 μM-KI and 50 mM-Bes[N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] adjusted to pH 7.0. The cells were incubated at 4°C or 37°C with 1 ml (3 cm dishes) or 0.5 ml (multwells) of binding medium containing ¹²⁵I-EGF at the required concentration. The test dishes also received the volume of pre-colostrum, colostrum or milk indicated in the Figure legends. After incubation, unbound radioactivity was removed by washing the cells four times with cold (4°C) phosphate-buffered saline (pH 7.4) containing 0.1% albumin and 0.1 μM-KI. The washed cells were extracted (60 min at 37°C) into 1 ml of 0.5 M-NaOH, and cell-associated radioactivity was determined in a gamma counter. Non-specific ¹²⁵I-EGF binding measured as cell-associated radioactivity in the presence of unlabelled EGF (2 μg/ml) was less than 5% of the total. Cell numbers were obtained from replicate dishes of cells treated with trypsin/EDTA as described above.

Assay for the stimulation of DNA synthesis in quiescent 3T3 cells

Portions of test agents were added directly to the depleted growth medium of quiescent cells. [³H]-Thymidine (20 μl) was added per dish to give a final concentration of 1 μCi/ml (1 x 10⁻⁶ M). After incubation for 40 h at 37°C, the medium was removed from the dishes and the cells were washed twice with cold phosphate-buffered saline (pH 7.4). The cells were extracted for 30 min with 1–2 ml of cold 5% (w/v) trichloroacetic acid, rinsed with ethanol, and air dried. The cells were dissolved in 1 ml of 0.1 M-NaOH and 0.5 ml of this solution was mixed with 10 ml of acidified scintillation fluid for counting of ³H.

Results

A mitogen in goat mammary secretions inhibits EGF binding to 3T3 cells

Goat pre-colostrum or colostrum (4%, v/v) caused an 80% inhibition of the binding of ¹²⁵I-EGF to 3T3 cells (Fig. 1). In contrast, 4% (v/v) milk obtained from the same animal 1 day post-partum caused only 8% inhibition of binding (Fig. 1). The ability of mammary secretions to stimulate DNA synthesis in quiescent 3T3 cells was studied with a similar pattern, i.e. high activity levels in pre-colostrum and colostrum, low activity levels in milk (Fig. 1). When lactogenesis was initiated prematurely by pre-partum milking, the levels of cell growth-promoting activity and the levels of ¹²⁵I-EGF-inhibiting activity in mammary secretions showed a parallel decline before parturition (Fig. 1). The results presented in Fig. 1 were obtained using defatted samples (see the Materials and methods section). Tests on representative defatted and decaseinated samples produced quantitatively similar results (not shown) indicating that the growth-promoting and the ¹²⁵I-EGF-inhibitory activity are present in the aqueous, non-casein (i.e. whey) fraction of the secretions. Treatment with proteolytic enzymes (trypsin plus chymotrypsin, both at 0.5 mg/ml final concentration, for 4 h at 37°C) or reducing agents led to a complete loss of both growth-promoting and ¹²⁵I-EGF-inhibiting activities. In contrast, heating whey to 100°C for 2 min caused only a small (approx. 1983

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Fig. 1. **Stimulation of DNA synthesis (open symbols) and inhibition of $^{125}$I-EGF binding (closed symbols)** in Swiss 3T3 cells by goat mammary secretions

Samples of secretions were obtained from goats 668 (squares) and 390 (circles) on the days indicated relative to parturition (day 0). The pre-partum samples from 668 were kept to a minimal volume (2–3 ml) to avoid initiating lactogenesis. Starting 8 days pre-partum, goat 390 was handmilked twice daily to remove all secretion from the gland cistern. This procedure led to a rapid initiation of lactogenesis prior to parturition. $^{125}$I-EGF binding assay: quiescent cultures of 3T3 cells were incubated with 0.5 ml of binding medium containing $^{125}$I-EGF (1 ng/ml) (41 000 c.p.m./ng) and 40 $\mu$l of mammary secretions/ml. After 90 min at 37°C the cells were washed and cell-associated radioactivity was determined as described in the Materials and methods section. Each point represents the mean value ($n = 2$ or 3) for the percentage inhibition of $^{125}$I-EGF binding relative to the binding to untreated, control cells. DNA synthesis assay: mammary secretions (25 $\mu$l/ml of medium) and $[^3]$H]thymidine were added directly to the depleted growth medium of quiescent cultures of 3T3 cells grown on 3 cm dishes. After a 40 h incubation at 37°C, the incorporation of radioactivity into acid-precipitable material was measured as described in the Materials and methods section. Each point represents the mean value for duplicate determinations of $[^3]$H]thymidine incorporation. In this experiment the addition of fresh calf serum to the medium (10%, v/v) produced an incorporation of 3.16 $\times$ 10^5 c.p.m./dish.

15% loss in both activities and heating to 60°C for 30 min was without effect on either activity (results not shown).

A sample of colostrum was chromatographed on a Bio-Gel P-10 column. Fractions from the column were tested for their ability to stimulate DNA synthesis in quiescent 3T3 cells and for their ability to inhibit $^{125}$I-EGF binding to these cells. A peak of $^{125}$I-EGF-inhibiting activity was found to be co-eluted with the cell-growth-promoting activity in the column void volume (Fig. 2).

Fig. 2. **Bio-Gel P-10 chromatography of goat colostrum**

Colostrum was dialysed (Spectrapor 3) versus several changes of 0.02 M-ammonium formate (pH 5.6), centrifuged, lyophilized and redissolved (one-fifth of original volume) in phosphate-buffered saline, pH 7.4. Concentrated colostrum (0.2 ml) was applied to a column (15 cm x 0.7 cm) of Bio-Gel P-10 equilibrated and eluted with phosphate-buffered saline. Fractions (0.5 ml) were collected and assayed for their ability to stimulate DNA synthesis (O) and to inhibit $^{125}$I-EGF binding (●) in quiescent Swiss 3T3 cells. $^{125}$I-EGF binding was measured after a 90 min incubation at 37°C with 0.5 ml of binding medium containing $^{125}$I-EGF (1 ng/ml) (37 000 c.p.m./ng) and 100 $\mu$l of the column fractions/ml. Each point represents the mean value ($n = 3$) for $^{125}$I-EGF binding expressed as a percentage of the control $^{125}$I-EGF binding. The stimulation of 3T3 cell DNA synthesis by column fractions (100 $\mu$l/ml of growth medium) was measured as described in the Materials and methods section. Each point represents the mean value of duplicate determinations of $[^3]$H]thymidine incorporation. In this experiment the addition of fresh calf serum (10%, v/v) to the medium produced an incorporation of 4.03 $\times$ 10^5 c.p.m./well.
Table 1. Ion exchange and immunoaffinity chromatographic behaviour of $^{125}$I-EGF-inhibiting activity in goat colostrum

<table>
<thead>
<tr>
<th>Column</th>
<th>Inhibition of $^{125}$I-EGF binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE-52</td>
<td>Retained fraction 10</td>
</tr>
<tr>
<td></td>
<td>Unretained fraction 48</td>
</tr>
<tr>
<td>Anti-EGF IgG--</td>
<td>Retained fraction 9</td>
</tr>
<tr>
<td>Sepharose</td>
<td>Unretained fraction 51</td>
</tr>
<tr>
<td>CM-52</td>
<td>Retained fraction 70</td>
</tr>
<tr>
<td></td>
<td>Unretained fraction 18</td>
</tr>
</tbody>
</table>

These results indicate that pre-colostrum and colostrum contain high concentrations of a heat-stable mitogenic polypeptide which inhibits $^{125}$I-EGF binding to cultured 3T3 cells. However, the active material is eluted from a Bio-Gel P10 column well ahead of the mouse EGF elution position (Fig. 2). In addition, the colostral $^{125}$I-EGF-inhibiting activity does not react with antibodies raised against mouse EGF (Table 1) and is not adsorbed to anion-exchange cellulose under conditions (Savage & Cohen, 1972) where more than 90% of native mouse EGF is bound (Table 1). In contrast, essentially all the activity was retained by a cation-exchange column at pH 7 and could be desorbed from this column using high salt concentrations (Table 1). Thus the colostral activity is due to a basic polypeptide with $M_\text{r}$ > 20000. It therefore differs from mouse and human EGF which are acidic polypeptides of $M_\text{r}$ approx. 6000. Because of this difference we have investigated the inhibition of $^{125}$I-EGF binding to 3T3 cells by colostrum in more detail.

Characterization of the colostrum-mediated inhibition of EGF binding to 3T3 cells

**Time-course of binding.** The time course of $^{125}$I-EGF binding to 3T3 cells in the presence and absence of colostrum is shown in Fig. 3. In control cultures maximal binding was reached after a 60 min incubation at 37°C. Continued incubation with $^{125}$I-EGF led to a decrease in cell-associated radioactivity due to the internalization of the $^{125}$I-EGF-receptor complex and the subsequent degradation of intracellular $^{125}$I-EGF (Carpenter & Cohen, 1976; Brown et al., 1979b). Colostrum caused a marked reduction in 3T3 cell-associated $^{125}$I-EGF (Fig. 3). This reduction was evident as early as 4 min after the simultaneous addition of colostrum and $^{125}$I-EGF to the cells. This result indicates that colostrum reduces total cell-associated radioactivity by inhibiting the initial binding of $^{125}$I-EGF to the cells rather than by affecting a subsequent step in the cellular interaction with $^{125}$I-EGF. This conclusion is supported by the finding that, at peak control binding (60 min), colostrum produces an 82% inhibition in cell-
Cell growth factors in colostrum

associated radioactivity. At this time approx. 30% of 3T3 cell-associated radioactivity remains on the cell surface (Brown et al., 1979b; K. D. Brown, unpublished results) and thus an effect on internalization alone could not account for an inhibition of this magnitude.

Binding as a function of $^{125}$I-EGF concentration. The effect of increasing concentrations of $^{125}$I-EGF on the binding to 3T3 cells in the presence and absence of goat colostrum is shown in Fig. 4. The inhibition of $^{125}$I-EGF binding by colostrum is competitive with no change in maximal binding ($B_{\text{max}}$) but with a 4-fold decrease in the apparent affinity of binding. Thus, half-maximal binding was achieved at concentrations of $^{125}$I-EGF of $8.3 \times 10^{-10}$M and $3.3 \times 10^{-9}$M in the control and colostrum-treated cells respectively.

Dependence on colostrum concentration and influence of temperature. The dose–response curve for the inhibition of $^{125}$I-EGF binding by colostrum is shown in Fig. 5. At 37°C 2% (v/v) colostrum caused a 50% inhibition of $^{125}$I-EGF while 5% (v/v) colostrum inhibited binding by more than 80%. Quantitatively similar results (not shown) have been obtained with the colostrum from eight goats. In contrast to the marked inhibitory effect at 37°C, colostrum is a much less potent inhibitor of $^{125}$I-EGF binding at 4°C (Fig. 5). Thus the mechanisms of colostrum- and EGF-mediated inhibition of $^{125}$I-EGF binding must differ since native EGF is an equally effective inhibitor of $^{125}$I-EGF binding at 37°C and 4°C (Fig. 5). The inhibitory factor in colostrum appears to act by an indirect mechanism which involves a temperature-sensitive step.

Colostrum does not down-regulate EGF receptors. Incubation of cultured cells with EGF leads to a decreased capacity of the cells to rebind EGF. Biochemical and morphological studies have demonstrated that this down-regulation of binding is due to the endocytic internalization of the occupied EGF receptors (reviewed by Haigler & Cohen, 1979; Adamson & Rees, 1981). The following experiment was undertaken to determine whether exposure of 3T3 cells to colostrum, at a concentration which maximally inhibits $^{125}$I-EGF binding, also down regulates EGF receptors. 3T3 cells were pre-incubated with 5% (v/v) colostrum for periods up to 22h,
Fig. 6. Colostrum does not induce down-regulation of EGF receptors on Swiss 3T3 cells

Quiescent cell cultures were incubated (37°C) with 20 ng of EGF/ml (●) or 50 μl of colostrum/ml (■). At the indicated times the cultures were rinsed twice with binding medium and 1 ml of binding medium was added per dish. The dishes were incubated for 1h at 37°C to allow bound ligand to be removed from cell surface EGF receptors. The cultures were again rinsed twice with binding medium and 1 ml of binding medium containing 30 ng of 125I-EGF (52000 c.p.m./ng) was added per dish. Cell-associated radioactivity was measured after a 90 min incubation at 37°C as described in the Materials and methods section. Each point represents the mean value (n = 3) obtained for 125I-EGF binding expressed as a percentage of the mean control value for binding to untreated cells.

washed and binding of 125I-EGF was measured. Pretreatment with colostrum for 2–5 h caused an apparent 'up-regulation' in the number of EGF receptors with a maximal 30% increase in 125I-EGF binding after a 3.5 h exposure to colostrum (Fig. 6). After a 22 h incubation with colostrum the value for 125I-EGF binding had fallen to 90% of the binding of untreated cells (Fig. 6). In contrast, pretreatment of 3T3 cells with 125I-EGF led to a rapid, sustained reduction in the number of cellular EGF receptors (Fig. 6). Thus, although colostrum contains a potent inhibitor of 125I-EGF binding to 3T3 cells, this inhibitor, in contrast with EGF, is unable to promote internalization and down-regulation of EGF receptors.

Discussion

Our results demonstrate that goat pre-colostrum and colostrum contain a growth factor for 3T3 cells which also inhibits 125I-EGF binding to these cells. The factor appears to be a basic polypeptide of Mr > 20000 and is thus quite different from human and mouse EGF. Furthermore, the characteristics of the inhibition of 125I-EGF binding by colostrum differ from that produced by native EGF. Colostrum-induced inhibition is temperature-dependent whereas EGF-mediated inhibition is temperature-independent. Colostrum, at concentrations which maximally inhibit 125I-EGF binding, fails to induce down-regulation of 3T3 cell EGF receptors even after a 22h incubation with the cells. Indeed, the initial effect of pretreatment with colostrum is to increase 125I-EGF binding (Fig. 6). The addition of colostrum to the growth medium of quiescent cells causes an early decrease in protein degradation (Ballard et al., 1982), an increase in protein synthesis, and an increase in cell size (K.D. Brown, unpublished results). This stimulation may give rise to an increase in the synthesis and/or insertion of plasma membrane receptors and may account for the apparent 'up-regulation' in the number of EGF receptors per cell. The subsequent decrease in cellular EGF receptors at 22h (Fig. 6) may reflect a cell-cycle-dependent reduction in EGF receptors as the colostrum-stimulated cells progress through the cell cycle.

The inhibition of 125I-EGF binding could be due to the binding of ligand by a protein in colostrum. However, if this is the case, the binding protein appears to be a growth factor since the growth-promoting activity and the 125I-EGF inhibitory activity co-purify through ion exchange and gel filtration and show similar resistance to heat denaturation and similar susceptibility to inactivation by reducing agents. Furthermore, 125I-EGF was eluted from a Sephadex G-50 column (equilibrated and eluted with binding medium) at the same position in the presence or absence of 4% (v/v) colostrum (results not shown) indicating that the radioactive ligand is not adsorbed to a colostral binding protein.

Recently, several chemically diverse growth-promoting agents have been found to inhibit 125I-EGF binding to Swiss 3T3 cells by an indirect mechanism involving a temperature-dependent reduction of receptor affinity. These include the tumour-promoting phorbol ester TPA (Brown et al., 1979a), the neurohypophyseal hormone, vasopressin (Rozengurt et al., 1981), and fibroblast-derived growth factor isolated from the conditioned medium of SV40 BHK cells (Rozengurt et al., 1982). The nature of the processes involved in this indirect inhibition is not known. The inhibitory effect does not require an intact cytoskeleton, protein synthesis, energy metabolism or the maintenance of ion gradients across the plasma membrane (Rozengurt et al., 1982). The effect is, however, markedly reduced at low temperature and it may be that a temperature-dependent lateral movement
of receptor proteins within the plasma membrane is required to establish the inhibition. The results presented in this work suggest that inhibition of $^{125}$I-EGF binding by the colostral factor is mediated by a similar mechanism.

The indirect nature of the inhibition of EGF binding by the colostral factor, together with the observation that the factor has biochemical characteristics quite different from those of mouse and human EGF, indicate that this factor is not an EGF-related polypeptide. The cell growth-promoting properties of human colostrum have not yet been investigated but human milk contains cell-growth-stimulating activity (Klagsbrun, 1978) which appears to be due, primarily, to its EGF content (Carpenter, 1980). In contrast, although goat, cow and sheep pre-colostrum and colostrum contain high levels of cell growth-promoting activity, milk from these species contains little or no activity (Klagsbrun & Neumann, 1979; Brown & Blakeley, 1982). Furthermore, we have now found that the major growth-promoting activity in goat colostrum is due to a factor apparently unrelated to EGF. Thus, our results indicate that both qualitative and quantitative species differences exist in the growth factor content of mammary secretions.

The origin of colostrum growth factor(s) is unknown. The high levels in pre-colostrum and colostrum, together with the rapid fall in levels at lactogenesis, are characteristic of the concentration of several blood-derived proteins in mammary secretions (McClelland et al., 1978; Healy et al., 1980). Although synthesis of the factor by the mammary gland cannot be excluded, it is probable that the colostral factor is transferred from the blood across the mammary epithelium. The pathway for this transfer could be paracellular, i.e., between the secretory cells. Alternatively a transcellular mechanism may operate whereby the factor is bound to receptors on the baso-lateral membrane, internalized by the epithelial cells and subsequently secreted across the apical membrane into the alveolar lumen. It is now well established that a wide variety of cell types bind and internalize various polypeptide hormones and growth factors, including EGF (Haigler & Cohen, 1979; Adamson & Rees, 1981). This internalization leads to the lysosomal degradation of the factor. However, it is conceivable that in secretory epithelia a modification of this pathway could permit the passage of an undegraded growth factor through the cell. We have infused $^{125}$I-EGF close-arterially into the mammary gland of goats and have found that intact, immunoreactive $^{125}$I-EGF appears in the mammary secretions after a lag of 30–35 min (Blakeley et al., 1982). Further experiments are in progress to investigate the mechanism of this transfer.

The physiological role of cell growth-promoting agents in mammary secretions remains to be determined. Polypeptide growth factors, including EGF, have been shown to influence the growth and development of mammary tissue in vitro (Topper & Freeman, 1980; Salomon et al., 1981), and the factor may be involved in the growth and development of the mammary gland prior to parturition and lactogenesis (Knight & Peaker, 1982). Alternatively, the factor may be involved in neonatal growth and development. Widdowson et al. (1976) found that the small intestine of piglets fed sow colostrum was markedly increased in weight and length compared with that of piglets fed water during the first 24 h post partum. Heird & Hansen (1977) found that small intestinal mucosal mass, DNA and protein content and enzyme activity increased in response to ingestion of colostrum when compared with an intake of an isocaloric simulated milk. These observations raise the possibility that growth factors in mammary secretions may be involved in controlling the early growth and maturation of the epithelial cells of the neonate’s intestine.

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References

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