ISOLATION AND IDENTIFICATION OF A SOUTH AFRICAN LENTIVIRUS FROM JAAGSIEKTE LUNGS

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ABSTRACT

In the course of attempts to grow the jaagsiekte retrovirus in cell culture, a typical lentivirus was isolated for the first time in South Africa from adenomatous lungs. Morphologically the virus could not be distinguished from other lentiviruses, but serologically it was shown to be more closely related to visna virus than to caprine arthritis-encephalitis virus. However, a preliminary restriction enzyme analysis of the linear proviral DNA of this new lentivirus (SA-DMVV) revealed that it is significantly distinct from visna virus and CAEV and therefore may represent a third type of lentivirus. Antibodies to the virus were demonstrated in a number of sheep in various parts of the country, but a direct link to a disease condition was not found. Attempts to produce lung lesions by intratracheal injection of the virus have been unsuccessful to date but a transient arthritis was produced by intra-articular inoculation. Viral replication seems to be enhanced in jaagsiekte lungs.

INTRODUCTION
Jaagsiekte (JS) or ovine pulmonary adenomatosis has been closely associated with maedi, a chronic progressive interstitial pneumonia, in various parts of the world. The two diseases were introduced into Iceland at the same time and possibly by the same imported animal (Paslos, 1976). In Peru, both diseases were demonstrated in one sheep (Snyder, DeMartini, Ameghino & Caletti, 1983), while both were shown to be present in the same flock in Kenya (Wandera, 1971), the Netherlands (Houwers & Terpstra, 1984) and in England (Markson, Spence & Dawson, 1983).

In South Africa, maedi and the associated neurological affection, visna, in their classical forms have never been diagnosed. However, in two early descriptions of the histopathological lesions of jaagsiekte, reference was made to ‘chronic catarhal pneumonia’ and ‘interstitial fibroid changes’ (Mitchell, 1915) as well as to ‘thickening of interalveolar tissue’ and ‘lymphoid infiltration’ (Cowdry, 1925), lesions which are certainly not typical of jaagsiekte. In comparing his own observations with those of these authors, De Kock as far back as 1929 concluded that there may be two specific lung diseases in sheep, a ‘papilliform cystadenoma’ (jaagsiekte) and a ‘chronic indurative catarrhal pneumonia’ (visna), in their classical forms having never been closely associated with maedi, a chronic progressive interstitial pneumonia, in various parts of the world. The two diseases were introduced into Iceland at the same time and possibly by the same imported animal (Paslos, 1976). In Peru, both diseases were demonstrated in one sheep (Snyder, DeMartini, Ameghino & Caletti, 1983), while both were shown to be present in the same flock in Kenya (Wandera, 1971), the Netherlands (Houwers & Terpstra, 1984) and in England (Markson, Spence & Dawson, 1983).

In South Africa, maedi and the associated neurological affection, visna, in their classical forms have never been diagnosed. However, in two early descriptions of the histopathological lesions of jaagsiekte, reference was made to ‘chronic catarrhal pneumonia’ and ‘interstitial fibroid changes’ (Mitchell, 1915) as well as to ‘thickening of interalveolar tissue’ and ‘lymphoid infiltration’ (Cowdry, 1925), lesions which are certainly not typical of jaagsiekte. In comparing his own observations with those of these authors, De Kock as far back as 1929 concluded that there may be two specific lung diseases in sheep, a ‘papilliform cystadenoma’ (jaagsiekte) and a ‘chronic indurative catarrhal pneumonia’, which was called ‘Graaff-Reinet disease’ after the district in which it was first encountered. Graaff-Reinet disease has not been diagnosed since, however.

When the aetiological agent of jaagsiekte (JSRV) was shown to be a retrovirus (Verwoerd, Williamson & De Villiers, 1980), the question was immediately raised of a possible relationship to visna virus, which is the prototype of the Lentivirinae, a subfamily of the Retroviridae (Matthews, 1979).

The morphology and morphogenesis of JSRV were quite distinct, however, and no evidence for a serological relationship to visna virus (VV) was found (Verwoerd et al., 1980; Payne, Verwoerd & Garnett, 1983). Furthermore, the negative results of a limited serological survey supported the contention, based on clinical and pathological observations, that maediva disna did not occur in South Africa.

The isolation of a lentivirus from the lungs of experimental jaagsiekte cases, which is reported in this paper, was therefore quite unexpected and prompted an investigation of its relationship to the other known lentiviruses, the prototypes of which are visna virus and caprine arthritis-encephalitis virus (CAEV), as well as its possible role in disease.

MATERIALS AND METHODS
Cell cultures
Sheep choroid plexus (SCP) cells were prepared from foetal tissues, frozen away as primary cultures and subsequently used for virus isolation within 3–6 passages. Ovine foetal trachea (OFTR), ovine foetal turbinate (OFTU) and Himalayan tahr ovary (HTO) cell lines were obtained from Dr Lehmkuhl, Animal Disease Center, Ames, Iowa. All cell lines were maintained in Dulbecco’s minimal essential medium supplemented with high glucose, glutamine and 10% foetal calf serum.

Lung rinse pellets
Lung rinse pellets were prepared from both jaagsiekte and normal lungs as previously described (Verwoerd, Payne, York & Myer, 1983). Briefly, 1–2 litres of cold tissue culture medium without serum was poured into the trachea and decanted after a gentle massaging of the lungs. Cells were removed from the rinse fluid by low speed centrifugation and used for establishing macrophage and primary lung cell cultures. The supernatant was pelleted in a Spinco Ti 15 batch rotor at 30 000 rpm for 90 minutes. The pellet was collected, stored at −70 °C if necessary, and further purified by passing through a 30 × 1,6 cm column of Sephacryl S-1000 superfine (Pharmacia).

Infection of cells
Virus was isolated by either co-cultivating the macrophages obtained from the lungs with SCP cells, or by infecting the cells with the semi-purified lung rinse pellets. In the latter case the cells were pretreated for 1 hour with polybrene at 8 μg/ml before adding the purified pellet in medium containing the same concentration of polybrene. Adsorption was allowed to take place for 3–4 hours.

Infected cell cultures were monitored for virus replication by looking for cytopathic effects (cell fusion and rounding of cells) and by assaying the supernatant at weekly intervals for reverse transcriptase (RDP) activity. The RDP assay has been described previously (Verwoerd et al., 1983).

Polyacrylamide gel electrophoresis
RDP-positive supernatants were pelleted at 30 000 rpm for 90 min in a Spinco rotor 30 and the pellets dissociated by heating for 3 min in a boiling water bath in 2,5 % SDS, 5,0 % β-mercaptoethanol and 12,5 %
glycerol. Proteins were then fractionated electrophoretically in a 0.1% SDS-containing 12.5% polyacrylamide gel with a 4% stacking gel according to King & Laemmli (1971).

**Immunoblot assay**

Protein-SDS bands were transferred from the polyacrylamide gels to nitrocellulose sheets (S&S) in a Bio-Rad electroboblot apparatus in a 20% methanol containing tris-glycine transfer buffer at pH 8.3. Transfer was usually carried out at 0.45 amps for 4 hours at 4°C. For immunological detection of the transferred proteins, the nitrocellulose was cut into the desired strips and blocked in 50% horse serum in PBS for 1 hour with gentle rocking. The strips were then incubated overnight with the relevant primary antiserum diluted 1:20 in 50% horse serum. Excess antiserum was removed by rinsing 3 times in washing buffer (0.025% Tween 20 in PBS) and the strips then incubated for 1 hour in peroxidase-conjugated antibody against sheep IgG. After repeating the washing procedure, freshly prepared peroxidase substrate (0.06 g 4-chloro-1-naphthol in 20 ml methanol + 100 ml 1% PBS + 60 μl H₂O₂) was added. When the bands were clearly visible the reaction was stopped by immersing the strips into deionized water for 10 min and drying between filter paper.

In all the experiments low molecular mass markers (Pharmacia), stained with amido black, were run concurrently with the samples, which included positive serum/antigen controls as required.

**Antisera**

Sheep anti-visna virus serum was obtained from Dr D. J. Houwers, Lelystad, Netherlands, and used at a 1:50 dilution. Goat anti-Mason-Pfizer monkey virus p27 was supplied by the National Cancer Institute, Bethesda, Maryland and used at a 1:60 dilution. Goat anti-CAEV serum was obtained from Dr O. Narayan, Baltimore, USA and used at 1:100. Goat anti-sheep IgG was obtained from Dakopatts, Copenhagen, Denmark, and used at a dilution of 1:400.

**Restriction enzyme digestion, Southern analysis and molecular hybridization**

Unintegrated proviral DNAs were selectively extracted from lentivirus-infected cells by the Hirt procedure and digested with restriction enzymes. The digested and undigested DNAs were analyzed electrophoretically in 1% agarose gels, transferred to nitrocellulose, and hybridized to a 32P nick-translated DNA of lambda vis 109 visna virus insert as previously described (Querat, Barban, Saule, Filippi, Vigne, Russo & Vitu, 1984).

**Electron microscopy**

Samples were prepared for electron microscopy as described previously (Payne et al., 1983) and viewed in a Jeol transmission electron microscope.

**Immunoperoxidase staining technique**

An anti-visna virus peroxidase conjugate was made according to Avrameas (1969). Frozen sections of jaagsiekte lungs were fixed in 96% ethanol, blocked for 10 min with 3% bovine serum albumin in PBS and then incubated with the conjugate diluted 1/50 in 3% bovine serum albumin in PBS. After incubating for an hour at room temperature the sections were washed with 0.5% Tween 20 in PBS. The substrate (10 mg 3,3'-diaminobenzidine tetrahydrochloride in 20 ml of PBS containing 0.01% H₂O₂) was added to the sections for 10 min. The sections were washed under running tap water stained for 1 min with Haematoxylin and mounted.

Initial isolation

The original discovery of a South African lentivirus was made during attempts to cultivate jaagsiekte retrovirus (JSRV) in sheep choroid plexus (SCP) cells. A lung tissue pellet, which was obtained from an experimental case of jaagsiekte as previously described (Verwoerd et al., 1983), was purified by gel filtration as described under Materials and Methods, without freon pretreatment. Virus concentrations in the eluted fractions were determined by means of a reverse transcriptase (RDP) assay. A combination of RDP-positive peaks was used to infect polybrene-treated secondary SCP cells (see Materials and Methods). RDP-activity was first detected in cell culture supernatants on Day 17 and reached a peak

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on Day 41 after infection (Fig. 1). At this stage typical cytopathic effects were seen, consisting of syncitium formation and dying cells. The retrovirus, which is produced by these cells and was first thought to be JSRV, therefore has a very long replicative cycle, albeit quite variable in different cell types (see below).

**Identification as a lentivirus**

The first indication that the virus replicating in SCP cells was not JSRV was obtained by electron microscopic examination. The morphogenesis of the virus was quite distinct from that of JSRV (Payne et al., 1983) and resembled that described for visna virus. The virus budded with a crescent shaped core with no intermediate space (Fig. 2A) to form immature particles with an electron-dense layer immediately below the viral envelope (Fig. 2B). Mature particles were membrane-bound with eccentric cores and in some particles a clear intermediate layer was visible (Fig. 2C). Multinucleoid particles were also observed (Fig. 2D). On some negatively stained particles there appeared to be knobs on the surface, but on most particles there was no distinct surface structure (Fig. 2E). These particles were quite distinct from those described for JSRV.

The buoyant density of the virus, determined by isopycnic centrifugation in a sucrose gradient, was 1.15. This provided additional evidence that the virus rescued in SCP cells was different from the JSRV present in the lung fluid, which had a density of 1.175.

A serological relationship between the newly isolated virus and visna virus was first detected by means of an ELISA test (results not shown). As the various members of the lentivirus family could not be distinguished by this,
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technique, an immunoblot-assay was utilized for this purpose (see Materials and Methods). The results of an experiment in which the relationship between the new isolate, JSRV, visna virus and CAEV was studied, are shown in Fig. 3. A visna virus antiserum specific for the 2 internal proteins p28 and p16 cross reacts with 2 antigens of identical size in the new virus, but not with JSRV. CAEV antiserum reacts only with the p28 antigen. Likewise, a goat antiserum prepared against the new virus reacts with p28 and p16 in the homologous reaction but not with JSRV. An antiserum against the p30 protein of Mason-Pfizer monkey virus (MPMV), which was recently shown to react with a JSRV antigen (Sharp & Herring, 1983) does not react with either of the lentiviruses.

It is clear, therefore, that the new isolate, subsequently called South African ovine maedi-visna virus, isolate 1 (SA-OMVV-1) shares the p28 group-specific antigen with visna virus and CAEV and the p16 visna virus specific antigen (Querat et al., 1984), suggesting that SA-OMVV-1 is antigenically closer related to visna virus than to CAEV, with regard to these two internal proteins. A similar study carried out in France, using an immuno-precipitation technique and radio-labeled viral proteins, confirmed these results and indicated that the new lentivirus contains four viral proteins similar in size to those of visna virus, i.e. gp135, p28, p16 and p14 (Barban et al., in preparation).

The antigenic relationship between gp135 of SA-OMVV-1 and visna virus was not clearly resolved using either technique, however. A restriction enzyme analysis of the proviral DNAs present in lentivirus-infected cells revealed that the nucleic acid sequences of the new lentivirus are distinct from those of visna virus as well as from those of CAEV. Briefly, unintegrated proviral DNA from cells infected by various lentiviruses was extracted by the Hirt method, digested by restriction enzymes and electrophoresed in an agarose gel. After the transfer of DNA fragments onto a nitrocellulose filter, viral DNA was detected by hybridization with 32P cDNA representative of visna virus genome (strain K1514). Fig. 4 shows that unintegrated linear DNA of visna virus, CAEV (strain Cork), CAEV (strain Crawford, G63) and SA-OMVV-2 (isolated from the tumour cell line 15.4) are all ± 10 kilobase pairs (kbp) long (lanes U). However, after digestion with EcoRI (lanes E) or Hind III (lanes H), the fragments generated from the full-length linear DNAs were distinct for the four examined lentiviruses, suggesting that SA-OMVV is a novel virus distinct from visna virus as well as two strains of CAEV. Liquid hybridization experiments between viral genomic RNA extracted from the four viruses and 32P cDNA specific for each of them confirmed that the South African lentiviruses are clearly distinct from visna virus and CAEV. In contrast, the two strains of CAEV, which had dissimilar restriction enzyme maps, were found to be closely related by liquid hybridization (Barban et al., in preparation).

Subsequent isolations
To determine whether the first isolation of SA-OMVV was a chance event or not, further attempts were made to isolate the virus from experimental jaagsiekte cases, normal animals and a few animals with pneumonic symptoms. Initially, lung rinse pellets were inoculated onto SCP cells and the medium was assayed for RDP activity as described above, but it was soon found that cocultivation of lung macrophages or primary lung cultures with SCP-cells gave more consistent results. As Table 1 shows, positive isolations were made from 18/20 experimental jaagsiekte animals, including a few that did not develop tumours but had lesions of mild interstitial pneu-
monia. Five isolations were made from normal animals, one of which had been in contact with jaagsiekte cases for some months. Primary isolations were mostly made in SCP cells, but viral replication was also demonstrated in an SCP cell line (SCP-IIIB), an ovine foetal trachea (OFTR), ovine foetal turbinate (OFTU) and Himalayan tahr ovary (HTO) cell lines. The identity of the isolates was confirmed by detecting the p28 and p16 antigens by means of the immunoblot assay using SA-OMVV-1 antiserum (results not shown) and by means of electron microscopy (Fig. 5). No differences could be detected between the different isolates.

Lentivirus particles were also observed budding from cells in primary cultures (Fig. 5A) and co-cultures. In some cases, large numbers of mature lentivirus particles were observed in the vacuoles of these cells (Fig. 5B & Fig. 5C) and also associated with cell debris.

In vivo localization of lentivirus replication

It was possible to identify budding lentivirus particles electron microscopically in 3 jaagsiekte tumours. These particles appeared to be budding from macrophages into the alveolar lumen in close proximity to tumour cells (Fig. 6A). The infected macrophages had abnormal endoplasmic reticulum and rounded pseudopodia indicating that the virus was affecting the metabolism of these cells. Mature lentivirus particles were observed in macrophage vacuoles as well as in the alveolar lumina (Fig. 6B).
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FIG 7 Lentivirus antigen (brown staining) detected in a frozen jaagsiekte lung section using a direct immunoperoxidase technique

TABLE 1 Independent isolations of lentivirus from sheep lungs

<table>
<thead>
<tr>
<th>Source of animals</th>
<th>Lung lesions</th>
<th>Number of positives</th>
<th>Immunoblot positives</th>
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<tbody>
<tr>
<td></td>
<td>JS pneumonia</td>
<td>7/9</td>
<td>4/5</td>
</tr>
<tr>
<td></td>
<td>JS only</td>
<td>7/7</td>
<td>4/5</td>
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<tr>
<td></td>
<td>Pneumonia only</td>
<td>4/4</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>18/20</td>
<td>10/13</td>
</tr>
<tr>
<td>Field case of pneumonia</td>
<td>Chronic purulent pneumonia</td>
<td>0/1</td>
<td>—</td>
</tr>
<tr>
<td>Normal sheep:</td>
<td>None</td>
<td>1/2</td>
<td>NT</td>
</tr>
<tr>
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</tr>
<tr>
<td>No contact</td>
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The electron microscopy results were confirmed by a direct immunoperoxidase staining technique using anti-visna virus serum to detect lentivirus antigen in jaagsiekte lungs. Lentivirus antigen was localized in alveolar lumina in close association with macrophages and was often adjacent to tumour lesions (Fig. 7).

Distribution

The results of our isolation attempts suggested that SA-OMVV-1 has been co-transmitted with JSRV during the course of our studies on the aetiology of jaagsiekte. It was indeed also possible to rescue lentivirus from our 15.4 tumour cell line, (SA-OMVV-2, unpublished results), which was established in 1975 from a jaagsiekte case and formed the basis of our jaagsiekte transmission studies. It was not surprising, therefore, that 23/27 sera from our experimental jaagsiekte animals older than 2 months gave positive results when tested for antibodies to SA-OMVV, using the immunoblot assay (Table 2). To obtain some information on the distribution of the lentivirus outside our own flock, sera were collected from clinically normal sheep from various parts of the country and screened for antibodies to SA-OMVV in the same way. The results, which are summarized in Table 2, indicate that the virus is widely distributed, but its incidence on different farms can vary from 0–30%.

In a previous study a large number of goat sera were tested for antibodies to CAEV using immunodiffusion and ELISA techniques (Adams et al., 1984). The negative results of that study were confirmed for 10 of the sera which were also included in the present series. Although goats can be experimentally infected with SA-OMVV (see below), no antibodies have so far been detected in normal goat sera.

Pathogenicity

As was mentioned above, mild interstitial pneumonia was found histologically in experimental animals injected intratracheally with lung rinse material obtained from adenomatous lungs. Similar lesions were found in animals that developed tumours and in those that did not.

In an attempt to prove that these lesions were caused by the contaminating lentivirus, SA-OMVV, isolated and cultivated in cell culture as described above, was injected intratracheally into a group of 18 new-born lambs and 2 goat kids. No lesions were found in 5 lambs necropsied after 3 months or in 5 after 12 months, and no symptoms were observed after 2 years in the surviving animals, except for one sheep which developed a mild
arthrit in both carpal joints. No virus could be isolated from the synovial fluid, however. All the animals showed a positive seroconversion.

To explore further the possibility that SA-OMVV may play a role in ovine arthritis analogous to that of CAEV in goats, 2 lambs and 2 kids were injected with 10^6 RDP units of SA-OMVV into the left carpal joint. One animal in each group developed an acute arthritis with considerable swelling after 6 weeks (Fig. 8). The condition persisted for about 3 weeks and then disappeared before virus isolation was attempted.

DISCUSSION

The isolation of a lentivirus from adenomatous lungs is not too surprising in view of the known association between jaagsiekte and maedi in various countries (Malmquist, Krauss, Moulton & Vander, 1972; Cutlip & Young, 1982; Irving, Perk, Hod, Gazit, Yaniv, Zimber & Tal, 1984). It was unexpected, though, because maedi or the related progressive pneumonia syndrome as found elsewhere had not been diagnosed in South Africa with the possible exception of the so-called 'Graaff-Reinet disease', described more than half a century ago.

The nature of the association between the 2 diseases is not known. Unlike experimental visna virus infections, where virus replication is restricted in vivo (Geballe, Ventura, Stowring & Haase, 1985), in animals infected with both jaagsiekte and lentivirus there appears to be active replication of the lentivirus in the lungs. It was recently demonstrated that visna virus can be transmitted efficiently to a natural case of jaagsiekte (Dawson, Vanables & Jenkins, 1985) suggesting that adenomatous lungs are more susceptible to the infection than normal lungs. These authors also demonstrated efficient spread of visna virus from this case to other animals, suggesting active virus production. This could be a manifestation of the known susceptibility of jaagsiekte lungs to various secondary infections. An interesting speculation in this regard is the possibility that JSRV may have an immunosuppressive effect, similar to that of the Mason-Pfizer monkey virus to which it is related. Another possibility is related to the known accumulation of macrophages in jaagsiekte lungs (Tustin, 1969). Lentiviruses were found to occur in a latent form in monocotes and to be activated during maturation of these cells to macrophages (Narayan, Kennedy-Stoskopf, Sheffer, Griffin & Clemens, 1983). Our demonstration that SA-OMVV can best be isolated from the lungs of 1-week-old and 2-week-old calves suggests a similar site for its replication, and may explain why adenomatous lungs seem to be particularly susceptible to the virus.

A third possibility is based on the demonstration that lentiviruses can be naturally present in a latent form in long term ovine fibroblasts and can be induced by super-infection with CAEV (Barban, Queurat, Sauze, Filippi, Vigne, Russo & Vitu, 1984). It is conceivable that JSRV could play a similar role in vivo in activating a latent lentivirus. Whatever the explanation for the apparent synergism between SA-OMVV and JSRV, the existence of such a phenomenon is supported by our observation of mild interstitial pneumatic lesions in co-infected lungs and our inability to produce similar lesions experimentally by lentivirus infection only.

The physical characteristics of the isolated virus, such as size, density, morphology and morphogenesis are typical for lentiviruses. Unambiguous proof of its identity as a lentivirus was provided by the serological demonstration that it shares the p28 group-specific antigen with both visna virus and CAEV. A p16 antigen is shared only with MVV, suggesting a close relationship to this virus. However, Southern blot hybridization experiments indicated that SA-OMVV is distinct from visna virus and CAEV and thus could represent a third type within the lentivirus group (Barban et al., in preparation).

The origin of the isolated lentivirus is uncertain. Isolations were consistently made from experimentally produced JS cases. These animals were all injected with material obtained from previous cases, however, suggesting an efficient co-transmission with JSRV over a number of years. Antibodies to SA-OMVV-1 were indeed found in a number of the serially produced cases, and virus could be rescued from the 15.4 tumour cell line with which the serial transmission started. A limited serological survey proved that SA-OMVV infection is not limited to our experimental flock. However, the incidence of antibodies to the virus in the flocks we investigated varied between 0 and 30%, and positive reactors were geographically wide-spread.

No definite role in any disease of economic importance can be assigned to the virus at present. It seems to have a very low pathogenic potential, similar to some of the ovine progressive pneumonia viruses (PPV) isolated in the USA. The experimental production of arthritis may indicate a possible involvement with this condition in nature, but similar results have been obtained with CAEV (Banks, Adams, McGuire & Carlson, 1983) and PPV (Oliver, Gorham, Perryan & Spencer, 1981) in lambs. As has already been discussed, possible involvement in chronic pneumonia was only observed in conjunction with jaagsiekte.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the excellent technical assistance of Mr P. W. Oosthuizen, Miss M. M. Oosthuizen and Miss M. J. Breutelaar of the VRI. We would also like to thank Prof. R. C. Tustin for histopathological examinations and Dr D. J. Houwers and O. Narayan for providing antisera.

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