Replication-deficient recombinant adenoviruses expressing the human immunodeficiency virus Env antigen can induce both humoral and CTL immune responses in mice

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An effective vaccine against infection with human immunodeficiency virus type 1 (HIV-1) is thought likely to require both a humoral and a CTL immune response. A non-replicating adenovirus vector system has been developed that can induce both a humoral and CTL response to HIV-1 envelope in mice. It is demonstrated that the stimulatory tat/rev 5′ splice-donor site sequence is required for efficient expression of HIV-1 env by this adenovirus vector system. rev can be provided bicistronically or in trans to result in good expression of env in vitro. A humoral immune response was detected after two immunizations with a bicistronic recombinant adenovirus (RAd142). The response was dose dependent, 5 × 107 p.f.u. inducing a response in some, but not all, animals and 1 × 108 p.f.u. giving a consistent antibody response. However, CTLs were induced by the lower dose of virus and after only one immunization with the higher dose. A positive CTL response was also seen consistently when the two monocistronic adenoviruses (RAd501 expressing env and RAd46 expressing rev) were given together, although two immunizations were required to give approximately the same level of response as seen with the bicistronic virus. RAd501 on its own also gave a low CTL response when two immunizations were given. It is suggested that a lower level of env expression is required to produce a CTL response than a humoral response and that this non-replicating adenovirus vector is a good system for inducing CTL.

Introduction

Adenovirus vectors have considerable potential as delivery systems for recombinant virus vaccines (for reviews see Imler, 1995; Wilkinson, 1994). Their ability to generate CTL responses and mucosal immunity may be particularly significant for protection against human immunodeficiency virus (HIV) infection. HIV-1 envelope glycoproteins have been expressed in actively replicating adenovirus vectors and shown to induce neutralizing antibodies in animal models (Dewar et al., 1989; Chanda et al., 1990; Natuk et al., 1992, 1993; Lubeck et al., 1994). However, replication-deficient adenovirus vectors have attracted increasing attention because of their improved safety and containment characteristics and their ability to limit expression in the target cell to the gene encoding the vaccine antigen. A non-replicating vector, which nevertheless expresses large amounts of antigen in target cells, should provide a safe and effective vaccine-delivery system, combining the advantages of killed and live vaccines.

We have linked a replication-deficient adenovirus type 5 E1− vector with the powerful constitutive cytomegalovirus (CMV) immediate early (IE) promoter (McGrory et al., 1988; Wilkinson & Akrigg, 1992). This vector replicates only in a permissive cell line (e.g. 293) that supplies the E1 gene product in trans, but can be used to infect a wide variety of non-permissive cell types. A gene placed under the control of the IE promoter is expressed at high levels but other adenovirus proteins are not normally produced (Wilkinson & Akrigg, 1992). When the recombinant virus is injected into an animal,
the expressed protein can induce antibodies and protective immunity (Jacobs et al., 1992). Furthermore, CTL responses have been shown to be induced by this type of vector (Fooks et al., 1995).

We describe here the construction of a replication-deficient adenovirus expressing the envelope glycoprotein gene (env) of HIV-1 strain IIIB. Expression of HIV-1 env is a complex process requiring co-expression of the HIV-1 trans-activator gene rev, which interacts with a Rev-responsive element in the env coding sequences (Feinberg et al., 1986; Sodroski et al., 1986; Rosen et al., 1988). A bicistronic adenovirus vector was therefore constructed expressing rev and env in tandem. There is also evidence that env expression may be dependent on the presence of the tat/rev 5‘ splice-donor site (SSD), even though splicing does not occur at this site (Chang & Sharp, 1989; Lu et al., 1990). However, other studies have suggested that this splice site is not required (Natuk et al., 1992; Emerman et al., 1992; Nasioulas et al., 1994). We confirm that the SSD is required for efficient Rev-stimulated env expression in plasmids and the adenovirus vector. We also show that such recombinant adenovirus vectors can induce both humoral and cellular immune responses in mice.

Methods

Cells, viruses and media. Primary human lung fibroblasts (MRC5 cells) and an adenovirus-transformed human embryonic kidney cell line, 293 (Graham et al., 1977), were grown in Dulbecco’s modified minimal essential medium (DMEM) containing 10% foetal calf serum (FCS) (ICN Biomedicals). All recombinant adenoviruses were based on Ad5dl309 (McGory et al., 1988) and were cultured in 293 cells.

Construction of recombinant plasmids and adenoviruses. Cloning of plasmid DNA fragments was performed by using standard methods (Sambrook et al., 1989) and checked by restriction endonuclease analysis. Plasmid DNA was extracted and purified by using QiaGen columns. The HIV-1 env gene from strain IIIB clone BH10 (Ratner et al., 1985) was inserted into a plasmid under the control of the CMV major IE promoter and polyadenylation signal sequence, as well as primers to detect adenovirus sequences (Zhang et al., 1993).

Transfections. Plasmid DNA was transfected into 293 cells (5 x 10⁶ cells per 25 cm² flask) by using Transfectam (Promega) according to the manufacturer’s instructions. The transfection mixture was applied to the cells in serum-free DMEM for 6 h at 37 °C and then 5 ml fresh DMEM plus FCS was added. Samples contained 5 µg DNA, comprising 3.5 µg test plasmid, 1.5 µg pMV19 (rev expression vector) or pUC19 control vector and 1 µg pLEp-gal (containing a β-galactosidase gene as an internal control for variations in transfection efficiency) (Wilkinson & Akrigg, 1992). pMV19 was constructed by removing the intron from a complete rev gene (HIV-1 IIIB strain) by using a Muta-Gene in vitro mutagenesis kit (Bio-Rad) and placing it under the control of the IE promoter of CMV. rev expression by this construct was confirmed by immunofluorescence (unpublished results) and biological activity was also demonstrated by enhanced env gene expression in co-transfection experiments. After incubation for a further 2 days at 37 °C, the cells were washed with PBS and harvested by scraping into PBS, centrifuged and lysed by resuspending in 120 µl PBS containing 10% Triton X-100 (BDH). Lysates were centrifuged (Eppendorf) to remove particulate matter and 100 µl of each supernatant was assayed for Env gp160/120 by ELISA (see below) and 20 µl was assayed for β-galactosidase activity using o-nitrophenyl galactoside (Sigma) as described previously (Wilkinson & Akrigg, 1992).

Adenovirus infections. Recombinant adenoviruses were plaque-purified three times by growth in 293 cells. To measure env gene expression, titrated virus was applied to MRC5 cells (1 x 10⁶ cells per 6 cm Petri dish) in 1.5 ml DMEM containing 10% FCS. Adenovirus–env recombinants were applied at an m.o.i. of 30 and RAd46 (rev recombinant) at an m.o.i. of 10. After incubation for 4 h at 37 °C, 5 ml DMEM containing 10% FCS was added and incubation was continued for 3 days at 37 °C. Cells were then washed with PBS and lysed with Triton X-100 as described above. Triton X-100 was used to lyse the cells, releasing cellular proteins including HIV-1 Env synthesized by the recombinant adenovirus, but does not affect the virus.

Larger-scale virus preparations were made for electrophoresis and blotting. 3.5 x 10⁵ 293 cells in a 175 cm² flask were incubated with each recombinant adenovirus at an m.o.i. of 1 until cells were released from the flask surface. Infected cells were harvested by centrifugation and lysed with 1.5 ml Triton X-100 in PBS.

To produce virus for inoculation of mice, ten 175 cm² flasks of 293 cells were incubated with each recombinant adenovirus at an m.o.i. of 1 until extensive CPE was seen and cells had been released from the flask surface. The supernatant was cleared of cellular debris by centrifugation (10 min, 12,000 g) and a virus pellet was obtained by centrifugation (25,000 r.p.m. for 2.5 h in a Sorval AH 629 rotor). The virus pellet was resuspended in PBS and a small aliquot was used in a plaque assay to determine the virus titre.

ELISA for env gene expression. The ELISAs were performed essentially as described by Jones et al. (1995). Each well of a 96 well plate (Nunc maxisorb) was coated with 50 µl of 1 µg/ml Galanthus nivalis lectin (Sigma) in PBS at 4 °C overnight. After washing with PBS containing 0.05% Tween 20 (Sigma), wells were blocked with 100 µl PBS containing 5% dried milk (Marvel) and 0.05% Tween 1 h at 37 °C. Plates were washed as before and a twofold dilution series of 50 µl test samples diluted in PBS was applied across the plate. After incubation for 2 h at 37 °C and washing, 50 µl of a 1:100 dilution of rabbit anti-HIV gp120 in blocking buffer was applied to each well and the plates were incubated for 1 h at 37 °C. Plates were washed and bound antibody was detected with 20 µl of a 1:5000 dilution of affinity-purified, peroxidase-conjugated donkey anti-rabbit immunoglobulin (Jackson Immuno-Research Labs) in 5 µl PBS containing 1% Tween 20 (Sigma), wells were blocked with 100 µl PBS containing 5% dried milk (Marvel) and 0.05% Tween.
blocking buffer. After incubation for 1 h at 37 °C, the plates were washed. Bound peroxidase was detected with 50 µl TM Blue substrate (Sigma), the plates were incubated for 15 min at 20 °C and the reaction was stopped by adding 50 µl 1 M H₃SO₄. The concentration of bound gp120 was estimated by comparing the A₄₅₀ with standard samples of known gp120 concentration (a generous gift from D. Jones, CAMR, Porton Down, UK).

### SDS–PAGE and Western blotting

Triton X-100 lysates of 293 cells infected with recombinant adenoviruses were clarified by centrifugation (Eppendorf) for 20 min. Supernatants were subjected to SDS–PAGE and electroblotted onto nitrocellulose filters (Amersham) as described by Polansky et al. (1997). Blots were blocked with 10% FCS in PBS containing 0.05% Tween 20 and probed with the same anti-gp120 and peroxidase-linked antibodies used for the ELISA. Proteins were visualized by using enhanced chemiluminescence (ECL, Amersham).

### Animals and administration of viruses

BALB/c mice (4–6 weeks old) were inoculated intraperitoneally (i.p.) with either 5 × 10⁵ or 1 × 10⁶ p.f.u. of the appropriate adenoviruses in 500 µl PBS: RAd142, RAd501 or RAd501 plus RAd46 were used. Mice were inoculated at the times detailed in each experiment.

### ELISA for anti-gp120 antibody production

Assays were performed in flat-bottomed microtitre plates (Nunc maxisorb). Plates were coated with 50 µl of each purified HIV-1 GB8 gp120 (a generous gift from D. Jones, CAMR) or purified, baculovirus-produced recombinant HIV-1 IIIB gp120 (obtained from the ARP programme EVA, NIBSC, UK). The antigen was diluted in bicarbonate coating buffer to 1 µg/ml and incubated overnight at 37 °C. Plates were washed with wash buffer (PBS containing 0.05% Tween) and wells were blocked with 60 µl wash buffer containing 5% dried milk powder (Marvel) for 1 h at 37 °C. Plasma samples were titrated as twofold serial dilutions across the plate and the antibody was allowed to bind for 2 h at 37 °C. The plate was washed five times before adding 50 µl of a 1:500 dilution of horseradish peroxidase-conjugated protein G. After 30 min incubation, the plate was washed and the bound peroxidase was detected with 50 µl of a 1:500 dilution of horseradish peroxidase-conjugated protein G. After 30 min incubation, the plate was washed and the bound peroxidase was detected with 50 µl TM Blue substrate (Sigma), the plates were incubated for 15 min at 20 °C and the reaction was stopped by adding 50 µl 1 M H₃SO₄. Antibody levels were estimated by measuring the A₄₅₀. All assays included a positive antisemur produced in rabbit as a control.

### CTL assays

In vitro expansion of precursor (p) CTL. HIV-1 IIIB Env glycoprotein contains an H-2D⁺-restricted CTL epitope, RGPGRAFVTI (Takahashi et al., 1993). The corresponding peptide was used to restimulate immune splenocytes in vitro as described previously (Hanke et al., 1998, a, b). Briefly, splenocytes from individual mice were isolated 14 or 28 days, respectively, after immunization with 10⁷ or 5 × 10⁷ p.f.u. and separately incubated in 10 ml lymphocyte medium (RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin 100 U/ml, 20 mM HEPES and 15 mM β-mercaptoethanol) in the presence of 2 µg/ml peptide for 5 days.

Target cells and ⁵¹Cr-release assay. On the day of assay, effector cells were washed three times, suspended in 10% FCS RPMI medium and, in duplicates, diluted twofold in U-bottomed wells (96 well plate; Costar) to give effector to target ratios of 100:1, 50:1 and 25:1. ⁵¹Cr-labelled P815 target cells (5000 cells) were added to the effector cells in medium containing 0.1 µM peptide and incubated at 37 °C for 5 h. Effector cells were also combined with targets in the absence of peptide to determine the percentage of no-peptide background lysis. Spontaneous and total ⁵¹Cr release were estimated from wells in which target cells were incubated in medium alone or with 5% Triton X-100. The percentage specific lysis was determined as 100 × (sample release — spontaneous release) / (total release — spontaneous release). To calculate the net specific release, no-peptide lysis was subtracted from the peptide-specific lysis. The spontaneous release was less than 10% of the total release.

### Results

**The tat/rev splice-donor site (5SD) is required for efficient expression of env**

(i) Transient transfection. Prior to construction of a recombinant adenovirus, the env open reading frame of HIV-1 strain IIIB contained in an Sspl–Xhol fragment (nt 5736–8473; Van Beveren et al., 1985) was cloned under the control of the promoter and polyadenylation signal of the CMV major IE gene (IEP- env). Expression of the recombinant plasmid was tested by co-transfection along with a plasmid pMV19 expressing the HIV-1 rev gene. env gene expression, as measured by ELISA on cell extracts with an anti-gp120 polyclonal antibody, was unexpectedly low when compared with a similar plasmid IEP-rev-env, which expressed the rev and env genes in tandem (MstI III 5343–8473) (Fig. 1).

To identify possible cis-acting stimulatory sequences in IEP-rev-env that were absent in IEP-env, additional recombinant plasmids were constructed in which different lengths of sequence upstream of env were included. These were tested for gp120 expression in the same way (Fig. 1). When co-transfected with pMV19, IEP-AfIII-env (nt 5634–8473) also expressed small amounts of gp120, but extending the sequence upstream by a further 48 nucleotides to nt 5586 (EcoNI) yielded a plasmid (IEP-5SD-env) that expressed gp120 efficiently when co-transfected with pMV19. This 48 bp sequence included the 5SD site at nt 5625 (Arya et al., 1985).

To test the ability of the 5SD alone to stimulate Rev-dependent env expression, a short oligonucleotide including the 5SD was synthesized and cloned adjacent to the Sspl site (nt 5736) in IEP-env to give IEP-oligo-env. The presence of the 5SD sequence restored gp120 expression completely to a level exceeding that of the bicistronic construct IEP-rev-env (Fig. 1).

(ii) Expression from recombinant adenoviruses. To determine whether the 5SD would exert a similar effect on env expression in the context of an adenovirus genome, recombinant adenoviruses Aden-R, RAd501 and RAd142 were constructed by using the IEP-env, IEP-5SD-env and IEP-rev-env expression cassettes, respectively. When MRC5 cells were infected with each of the recombinant adenoviruses (Fig. 2a), if necessary co-infecting with RAd46 (rev) to supply rev in trans, only RAd501 and RAd142 (rev–env) produced gp120 efficiently. Therefore, the 5SD also appeared to be required for env expression in a different background (adenovirus) and in a non-immortalized cell line (MRC5).

To confirm the presence of gp120/160 in infected cell extracts, the same adenovirus constructs were used to infect 293 cells. Cells were harvested 3 days post-infection and cell lysates were electrophoresed on SDS–polyacrylamide gels.
Fig. 1. Expression of gp120 by plasmid constructs co-transfected into 293 cells. The length of genome upstream of the \textit{env} ATG included in the plasmid is indicated diagrammatically alongside the plasmid name. All constructs contained the downstream \textit{env} gene nt 5801–8473. Error bars represent standard deviations from the means of three experiments. The amounts of gp120 produced by each sample were normalized against the levels of \( \beta \)-galactosidase produced by the pIEP-gal plasmid.

Fig. 2. Expression of gp120 by adenovirus constructs. (a) Level of gp120 expression as measured by ELISA after infection of MRC5 cells. Error bars represent the standard deviations from the means of three experiments. (b) Western blot analysis of adenovirus-infected 293 cell extracts probed with an anti-HIV-1 Env polyclonal antibody.
Induction of immunity with defective adenovirus

Western blots of the gels were then immunostained with the same antibody against gp120. A band corresponding to gp160 was observed in the lanes containing extracts of cells infected with RAd142 and RAd501 + RAd46 (Fig. 2 b). Neither Adenov-R nor RAd46 alone yielded detectable gp160 or gp120. A 44 kDa species was detected in all lanes. Bands detected at approximately 35 kDa from RAd501 + RAd46- and RAd142-infected cells were also present in other lanes, as revealed by longer exposure (data not shown). These species were therefore unrelated to HIV Env.

Immunization with recombinant adenoviruses induces humoral immune responses

We first sought to determine whether RAd142 produced a humoral response after i.p. inoculation at 0 and 3 weeks of either a high dose (1 × 10⁸ p.f.u.) or a low dose (5 × 10⁷ p.f.u.) of virus. Antibody levels were measured at 2, 4 and 6 weeks. As shown in Fig. 3, 3/3 animals receiving the high dose of virus produced antibodies by week 4. Two of three animals receiving the low dose of virus had also responded, but produced lower titres.

A second experiment was carried out to see whether the antibody response produced by the low dose of virus (5 × 10⁷ p.f.u. RAd142) could be boosted by inoculation at 0, 3 and 6 weeks. Also, using the same inoculation regime, we sought to determine whether an immune response could be elicited by simultaneous i.p. inoculation with the separate adenoviruses RAd501 and RAd46 (5 × 10⁷ p.f.u. of each virus) or RAd501 alone. Five animals were inoculated in each group. Serum samples were taken to measure antibody levels at 0, 2, 4, 6 and 9 weeks. However, no significant level of antibody production was seen in any of these animals (data not shown). These animals were then sacrificed for CTL assays.

Further experiments using the high dose of virus were carried out, again using three groups of animals injected with RAd142, RAd501 or RAd501 + RAd46. Animals were inoculated i.p. with 1 × 10⁸ p.f.u. of each virus, either twice at weeks 0 and 3 with antibody levels being estimated at weeks 2, 4 and 5, or only once with antibodies being measured at week 2. Again, the RAd142 group of animals produced antibodies by week 4 (after two inoculations), giving a mean \( \log_{10} \) end-point titre of 2.51, but not at week 2 (after one inoculation). No significant antibody production was seen in either of the other two groups. Animals were also assayed for CTL responses.

Immunization with recombinant adenoviruses induces CTL responses

(i) Mice immunized three times with 5 × 10⁷ p.f.u. virus. Splenocytes from four mice immunized with RAd142 (rev-env)
had HIV Env-specific CTL activity, as did splenocytes from 4/5 mice immunized with RAd501 (env) + RAd46 (rev), although the responses were weaker in two of these animals than those seen in RAd142-immunized animals. One mouse immunized with RAd501 alone had a weak response. The mean activity for each group of responders is shown in Fig. 4. One mouse in each of the RAd142 and RAd501 groups died, for reasons unknown, before the end of this experiment, but it was thought unlikely to be related to the immunization.

(ii) Mice immunized with either one or two doses of 1 × 10^8 p.f.u. virus. All mice receiving RAd142 produced an HIV Env-specific CTL response, the highest level being seen in those receiving two doses of virus. HIV Env-specific CTL were also induced after the co-administration of RAd501 and RAd46. A single immunization with 1 × 10^8 p.f.u. of each virus induced CTL activity that was lower than that seen with a single immunization with RAd142, but after two immunizations, HIV Env-specific CTL were induced at a similar high level. Immunization with a single dose of RAd501 alone induced a weak response in one animal only, whereas two immunizations of 1 × 10^8 p.f.u. RAd501 consistently induced an HIV Env-specific CTL response in all animals. This response was weak in four of five animals, ranging from 10 to 16% specific lysis at an effector to target ratio of 100:1. In one animal, specific lysis reached 39% at an effector to target ratio of 100:1, but this was still consistently lower than the activity seen in mice immunized with RAd142 alone or with a combination of RAd501 and RAd46.

Discussion

When plasmids were constructed with the HIV-1 env gene under the control of the CMV major IE promoter and transfected into 293 cells, little or no env expression was detected by ELISA for gp120 unless a short sequence (nt 5586–5634) immediately upstream of the u5 open reading frame was present. The stimulatory sequence allowed gp120/160 production when rev was provided either on a separate plasmid or as a bicistronic construct expressing rev and env in tandem. Significantly, this sequence contained the SSD site at nt 5625. Providing the SSD site in the form of a small synthetic oligonucleotide restored efficient expression of the env gene.

The presence of the 5SD sequence was also required for efficient expression of the env gene in the context of an adenovirus vector and in different cell types, although it was possible to obtain detectable levels of env expression without the 5SD site if MRC5 cells were infected with Adenv-R plus RAd46 at high m.o.i. for at least 6 days (data not shown). The mechanism by which the 5SD sequence regulates env gene expression is not understood, but seems to be linked either directly or indirectly to the mechanism of Rev trans-activation (Chang & Sharp, 1989; Lu et al., 1990; Hammarskjold et al., 1994; Kjems & Sharp, 1993; Barksdale & Baker, 1995; Mikaelian et al., 1996). We have shown that significant levels of HIV-1 gp120 can be produced by recombinant adenoviruses either by expressing rev bicistronically (RAd142) or by providing rev in trans on a separate adenovirus (RAd501 plus RAd46) when grown in vitro.

We next used these constructs in vivo to investigate their immunogenicity. It has been shown previously that a replication-deficient adenovirus expressing the lacZ gene can induce a long-term humoral and cellular response after a single inoculation (Juillard et al., 1995) and that protective immune responses can be obtained (Jacobs et al., 1992; Fooks et al., 1998) with the administration of some recombinant adenoviruses. It is clear from our studies that the bicistronic virus, RAd142, is immunogenic and can produce both humoral and CTL responses in mice. The humoral response required two inoculations and was dose dependent, requiring 1 × 10^8 p.f.u. to give a consistent response. Providing rev in trans was thought less likely to give a good immunogenic response, as this requires the simultaneous infection of any given cell by the two viruses. Others (Chenciner et al., 1997) have shown, however, that simultaneous infection with three recombinant adenoviruses, expressing the simian immunodeficiency virus (SIV) Env protein and the HIV-1 Tat and Rev proteins, can be successful in inducing anti-SIV Env antibodies. Dual infection with our two recombinant adenoviruses has not been found to be successful in producing an antibody response in any experiment carried out. We, however, used a lower level of each virus (1 × 10^8 p.f.u.) compared with that used by Chenciner et al. (1997) (1 × 10^9 p.f.u.), and this could have resulted in a smaller number of cells being doubly infected and thus a lower level of Env being produced. Simultaneous infection with our two adenoviruses did induce a CTL response, however, even when low doses of virus were used. A CTL response could also be detected after a single inoculation of adenovirus, whereas an antibody response always required two inoculations. This suggests that only a very low level of expression is required for the adenovirus to produce a CTL response compared with that required for a humoral response. The fact that RAd501 on its own could elicit a CTL response, albeit weak, supports the idea that only low-level expression of env is required. CTL responses in mice in the absence of serum IgG have also been described after mucosal administration of recombinant adenovirus expressing SIV p55 Gag from the E3 region (Flanagan et al., 1997). Taken together, these data suggest that it may be possible to manipulate the immune response to the transgene product by altering the dose of virus administered, the number of immunizations and the route of administration. Low doses of virus or single inoculations can produce a cellular response, whereas high levels of virus are required to elicit a humoral response as well. It should be noted, however, that the CTL responses were detected after expansion in vitro of pCTL, whereas humoral responses were analysed by detection of secreted product, i.e. antibody. Further work will reveal whether low doses of
recombinant adenovirus can prime B cells in the absence of antibody production. It is possible that good humoral responses could be obtained by using a combination vaccination strategy, such as priming with a recombinant adenovirus followed by an antigenic boost with an alternative vector or protein.

We were interested to observe that a CTL response was induced by using the trans-activated env construct. It has proved technically difficult to produce bicistronic adenovirus constructs, although the monostronic versions are relatively easy to construct. We have constructed several other monostronic env-recombinant adenoviruses that have been made by using field isolates from Uganda (representing clades A and D) and the clinical isolate W61D (clade B). Studies using these constructs expressing more relevant HIV-1 isolates will now be possible, including analysis of protective efficacy as cognate HIV-1 env–SIV chimeras (SHIVs) become available.

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References


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