Mucosal alloimmunization elicits T-cell proliferation, CC chemokines, CCR5 antibodies and inhibition of simian immunodeficiency virus infectivity

Lesley A. Bergmeier, Kaboutar Babaahmady, Yufei Wang and Thomas Lehner

Mucosal Immunology Unit, Guy’s, King’s and St Thomas’ Hospital Medical and Dental Schools, King’s College London, London SE1 9RT, UK

The hypothesis was tested that mucosal stimulation with unmatched mononuclear cells would induce systemic alloimmune responses. Rectal or vaginal mucosal administration of $10^4$–$10^7$ unmatched mononuclear cells induced significant dose-dependent T-cell proliferation stimulated by the allogeneic cells in rhesus macaques. This was associated with a significant upregulation of CD8+ T-cell-derived suppressor factor, as well as the CC chemokines CCL3, CCL4 and CCL5. In addition, there was a dose-dependent increase in antibodies to CCR5. These responses were associated with decreased in vitro simian immunodeficiency virus (SIV) infectivity of CD4+ T cells. A further investigation of SIV infectivity of CD4+ T cells separated from multiparous macaques also showed significant inhibition compared with male macaques. It is suggested that vaginal or rectal exposure to allogeneic stimulation by a partner’s HLA antigens in seminal fluid, as occurs during sexual intercourse, or immunization by semi-allogeneic fetuses in multiparous females may elicit protection against SIV or human immunodeficiency virus infection.

INTRODUCTION

Sexually transmitted diseases in general and human immunodeficiency virus (HIV) infection in particular are transmitted through the vaginal or rectal mucosa. A potentially important factor that has not yet been explored is the effect of HLA antigens in the cells found in ejaculates on the immune response of the partner. Ejaculates contain CD4+ T cells, macrophages, neutrophils, immature germ cells (Quayle et al., 1997), epithelial cells that express HLA antigens and cell-free HLA antigens (Koelman et al., 2000). Spermatozoa may express HLA class I and II antigens, though in a cyclical pattern (Martin-Villa et al., 1999). The possibility that HLA antigens in ejaculates might elicit an alloimmune response needs to be studied, especially as alloimmunization induces one of the most potent natural immune responses in which up to 10% of the T-cell repertoire is directed against HLA antigens (Blattman et al., 2002; Suchin et al., 2001).

To the best of our knowledge there is no recorded evidence that mucosal alloimmunity has been investigated. This is somewhat surprising in view of the common exposure of vaginal and rectal mucosa to alloantigens in ejaculates. However, we recently reported that unprotected sexual intercourse with a monogamous partner elicited a significant alloimmune response to the partner’s mononuclear cells, compared to unrelated cells (Peters et al., 2004). The CD4+ T cells from these women showed significant in vitro resistance to HIV-1 infection, compared with cells from those who practised protected sex.

Xenoimmunization of macaques with simian immunodeficiency virus (SIV) grown in human T cells has consistently been shown to protect the animals from SIV infection (Carlson et al., 1990; Desrosiers et al., 1989; Langlois et al., 1992; Murphey-Corb et al., 1989; Stott et al., 1990). There is also evidence that alloimmunization in macaques may protect them against SIV infection (Stott, 1994). Antibodies to class I and class II molecules have been found in macaques immunized (Bergmeier et al., 1994) or infected (Polyanskaya et al., 2003) with SIV. Xenoimmunization of macaques significantly increased the concentration of CD8-derived suppressor factor (CD8-SF), CCL5, CCL3 and CCL4 (RANTES, MIP-1α and MIP-1β, respectively), which are associated with protection against SIV infection (Wang et al., 1998). Furthermore, human in vitro studies suggest that alloantigens may induce HIV cross-reactive antibodies, cytotoxic lymphocytes or soluble factors (Bruhl et al., 1996; Clerici et al., 1993; Shearer et al., 1993). Systemic alloimmunization in women revealed that the three CC chemokines are significantly upregulated and that the CCR5 (CC chemokine receptor 5) and CXCR4 co-receptors are downmodulated (Wang et al., 1999a). Both M- and T-tropic HIV replication was inhibited in vitro by CD8-SF derived from peripheral blood mononuclear cells (PBMCs) of these women, in addition to a dose-dependent decrease in infectivity of CD4+ T cells.
Alloimmunization has been proposed as a strategy for inducing immune protection against HIV infection (Lehner et al., 2000a; Shearer et al., 1993). Epidemiological evidence suggests that sex workers in West Africa who appear to be resistant to HIV infection express rare HLA alleles (Celum et al., 1994). Transmission of HIV from mother to baby occurs more frequently among uniparous women (Kind, 1995) and mother–child HLA class I concordance increases perinatal HIV-1 transmission (MacDonald et al., 1998). Furthermore, selected sera from multiparous women showed significant CCR5 antibodies and in vitro inhibition of HIV-1 replication (Wang et al., 2002). On the basis of these findings, we postulated that vaginal or rectal exposure to HLA antigens in ejaculates might elicit mucosal alloimmunity, generating CD8− cell-derived antiviral factors and CC chemokines that affect immunity and transmission of the R5 strains of HIV.

The aim of this investigation was to determine in rhesus macaques whether rectal or vaginal mucosal exposure to allogeneic cells induced alloimmunity and affected SIV infectivity of CD4+ T cells. The results suggested that mucosal exposure to allogeneic cells in macaques elicited allogeneic T-cell proliferation, CD8+ cell-derived CC chemokines and antiviral factors, serum antibodies to CCR5 and a decrease in in vitro SIV infectivity of CD4+ T cells. However, major histocompatibility complex (MHC) typing of the macaques could not be done, because they were of Chinese origin for which typing reagents are not available, so the data should be interpreted with caution. In a further study of multiparous macaques in which natural alloimmunization may have been induced by the fetal semi-allogeneic cells, significant in vitro inhibition of SIV infectivity of CD4+ T cells was demonstrated, when compared with male macaques, as definitive nulliparous female macaques were not available.

**METHODS**

**Mucosal alloimmunization in rhesus macaques.** The experiment was designed to investigate non-traumatic rectal or vaginal immunization in eight rhesus macaques with alloimmune cells. A group of six male rhesus macaques, 4–7 kg in weight, were given PBMCs in RPMI 1640 from a single unmatched macaque by instillation into the rectum of 104, 106 or 107 PBMCs in a volume of 1 ml by means of a lubricated paediatric naso-gastric tube (Lehner et al., 1996). A control macaque was given 107 autologous PBMCs by the rectal route. Two female macaques were given 107 PBMCs in a volume of 0.5 ml, by the vaginal route, using a nasa-gastric tube. Immunization was boosted 1 month later. Venous blood was taken before and 1 month after each immunization. PBMCs were separated by density-gradient centrifugation on Ficoll (Nycomed) and enriched by panning method, as described previously (Lehner et al., 1996). CD4+ cells were removed using a monoclonal antibody (mAb) against CD4 (OKT4 hybridoma culture supernatant), and monocytes and B cells were removed using antibodies against immunoglobulin (Serotec). The cells (>85% CD8− T cells) were then stimulated for 3 days with 10 μg phytohaemagglutinin (PHA; Sigma) ml−1 in RPMI 1640 medium with 10% FCS, supplemented with 2 mM glutamine and 100 μg penicillin and streptomycin ml−1. PHA-stimulated CD8− blasts were then washed and resuspended at a concentration of 2 x 106 cells ml−1 in 10% FCS/RPMI 1640 medium containing 10% interleukin 2 preparation (IL2; Biotest UK). After 2 days of incubation at 37°C in an atmosphere of 5% CO2, the culture supernatant was collected and the cells replenished with fresh medium. This procedure was repeated up to three times. The collected supernatants were filtered through a 0.2-μm syringe filtration device and stored at −70°C for the CD8−SF activity assay.

**Assay of inhibition of SIV replication by CD8−SF.** Enriched CD4+ cells were prepared from simian PBMCs by negative selection using anti-CD4 mAb, as described above. CD4+ cells were stimulated for 3 days with 10 μg PHA ml−1 in 10% FCS/RPMI 1640. The cells were washed and pellets of 106 CD4+ cells were incubated with 100 μl SIVmac251 stock preparation containing 35 000 c.p.m. reverse transcriptase (RT) activity for 3 h. After incubation, free virus was washed off with culture medium and 2 x 105 cells per well were plated on to 96-well tissue culture plates (Costar). To assay the activity of CD8−SF, 100 μl CD8− cell-culture supernatant diluted 1:2 or 1:5 was added at the start of incubation to SIV-infected CD4+ cells. As a control, CD4+ cells were cultured in medium alone. After incubation for 2 days, 100 μl per well of the culture fluid was removed to monitor RT activity and replaced with 100 μl per well of diluted CD8− cell supernatant (1:2 or 1:5) or control medium. This was repeated every 2 days for up to 14 days and the RT activity was determined using Quanti-T-RT kits (Amersham).

**Assay of CC chemokines.** The CC chemokines CCL5 (RANTES), CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL2 (MCP-1) were assayed in the culture supernatants generated by PHA stimulation of CD8− T cells before and 1 month after each of the two alloimmunizations (Lehner et al., 1996). Specific ELISA capture assays (R&D Systems) were used for the CC chemokines and the results expressed in pg ml−1. Results are also presented for the combined concentration of CCL3, CCL4 and CCL5.

**Preparation of CCR5 in baculovirus and antibody assay by ELISA.** CCR5 was generated in baculovirus and expressed in an insect cell line, as described previously for SIV gp120 (Doyle et al.,...
Mucosal alloimmunization and SIV infectivity

1995). Briefly, high-titre (10⁹ p.f.u. ml⁻¹) baculovirus stock expressing CCR5 was used to infect insect cells for 2 days at 28°C. Cells were harvested and CCR5 isolated. A recombinant baculovirus expressing CCR5–His tag was prepared using the full-length gene of human CCR5, which was PCR cloned from pDNA3.1 CCR5 DNA (kindly provided by Dr John Moore, Department of Microbiology & Immunology, Weill Medical College of Cornell University, NY, USA) into the baculovirus transfer vector pACHis. The DNA construct was then used to form baculovirus expressing CCR5 with a six-His tag as described previously for SIV gp120 (Doyle et al., 1995). Expression of the gene was confirmed by PCR and by immunostaining of the infected cells. Purification of the protein through binding of the six-His tag to nickel-agarose was attempted but proved difficult under native conditions. Instead, we prepared CCR5 infected-cell lysates by three freeze–thaw cycles in 1 % Triton X-100/PBS. The purity of the baculovirus-prepared CCR5 has been demonstrated elsewhere (Lehner et al., 2001). Antibodies to CCR5 were detected by ELISA by coating plates with baculovirus expressing CCR5 and with baculovirus alone. The plates were incubated with doubling dilutions of the serum samples. Bound antibody was detected by incubation with rabbit anti-monkey IgG (2 μg ml⁻¹; Sigma), followed by affinity-purified goat anti-rabbit IgG–alkaline phosphatase conjugate (Sigma). IgG antibody titres are presented as reciprocals before and after each immunization.

SIV infectivity of CD4⁺ T cells in vitro. CD4⁺ T cells were enriched from PBMCs by depletion of CD8⁺ cells, using mouse anti-monkey CD8 mAb (GM9 culture supernatant from the hybridoma), followed by goat anti-mouse antibody (STAR 87; Serotec), using the panning technique (Lehner et al., 1996). CD4⁺ cells (3 x 10⁶ ml⁻¹) were activated with 10 μg PHA ml⁻¹ in 10% FCS/RPMI 1640 for 3 days, and then washed with medium and cultured in RPMI 1640 with 20 % IL2 (Biotest UK) overnight. Serial dilutions of SIVmac 251 were prepared, with 30 000 c.p.m. RT activity per 100 μl. Aliquots of 0·6 x 10⁶ cells were placed in universal tubes and infected with serial dilutions of SIV containing m.o.i. values of 10⁻⁷–10⁻⁴ for 2 h. Cells were washed three times with medium and then cultured in triplicate at a concentration of 2 x 10⁶ cells per well in 200 μl in 96-well culture plates. Every 3 days, 100 μl culture supernatant was replaced with 100 μl medium supplemented with 20 % IL2. On day 9, RT activity was assayed with the Quan-T-RT assay system (Amersham), using 70 μl cell-free culture supernatant per test. Results are presented as the mean (±SEM) of RT activity (c.p.m.) of three macaques before and after alloimmunization with 10⁶ cells.

SIV infectivity of CD4⁺ T cells in multiparous macaques. A group of five multiparous macaques was available, with recorded histories of three to five live births up to a year before their CD4⁺ T cells were examined for SIV infectivity. As we were unable to find nulliparous female macaques with a definitive history of no live or aborted fetuses, we used five macaques as controls instead. The SIV infectivity of their CD4⁺ T cells was evaluated as described above.

RESULTS

T-cell proliferative responses

In all eight macaques that were alloimmunized twice either rectally or vaginally, a rise in the T-cell proliferative response to the alloantigens was elicited from an SI value of <3 before immunization to SI values of between 6·8 and 18·2 (Fig. 1). After the first alloimmunization, 4/4 macaques immunized with 10⁷ cells yielded the highest SI (range 6·5–15·2), whereas 3/4 of those immunized with 10⁴ or 10⁵ cells yielded the lowest SI (range 1·0–3·0). The fourth macaque stimulated with 10⁴ cells showed a slightly raised pre-immunization SI of 3·5, which increased to 9·5 after the first immunization (Fig. 1). After the second alloimmunization, all macaques showed significantly raised SI values, in contrast to the control macaque immunized with 10⁷ autologous PBMCs, which showed no change in proliferation (SI value of 1·6 before and after immunization; Fig. 1). Both vaginal and rectal alloimmunizations elicited significant T-cell proliferative responses (SI > 6·0) and there was no obvious difference in the level of T-cell stimulation between the two mucosal routes. None of the mucosally alloimmunized macaques responded to stimulation by third-party cells from one macaque (data not shown). The results suggested that mucosally alloimmunized macaques could elicit secondary dose-dependent T-cell proliferative responses.

CD8-SF

The effect of culture supernatants derived from CD8⁺, enriched cells stimulated with PHA on the growth of SIV in CD4⁺-enriched cells was determined by RT activity. An increase in CD8-SF greater than 50 % was found in all but

Fig. 1. Effect of mucosal alloimmunization with unmatched mononuclear cells on T-cell proliferation. PBMCs from a single macaque were appliedatraumatically on to the surface of the rectal mucosa of six macaques, in doses of 10⁴ (△), 10⁵ (▲) and 10⁷ (●) cells, and on to the vaginal mucosa [10⁷ (○) cells] of two macaques. A control macaque was given 10⁷ (□) autologous PBMCs. Immunization was boosted 1 month later. A one-way mixed lymphocyte reaction was used to stimulate PBMCs with irradiated allogeneic PBMCs for 4 days. Results are expressed as stimulation indices.
one alloimmunized macaque (Fig. 2a). The mean (± SEM) pre-immunization level of CD8-SF at a 1:2 dilution was 29·9±7·2 %, compared with the post-immunization level of 69·4±7·2 %, and the corresponding values at a 1:5 dilution (not presented) were 15·4±4·7 and 36·9±9·9 %, respectively. These differences were significant at both dilutions of CD8-SF, as analysed by the paired Student’s t-test (t = 5·63, P = 0·001; t = 3·11, P = 0·02).

**CC chemokines generated from CD8-enriched cells**

CC chemokines were assayed in culture supernatants, generated by stimulating CD8-enriched cells with PHA, as described previously (Lehner et al., 1996; Mackewicz & Levy, 1992). The combined concentration of CCL3, CCL4 and CCL5 varied between 2652 and 4296 pg ml⁻¹ before immunization and rose to between 5453 and 14 466 pg ml⁻¹ after the second immunization (Fig. 2b). Alloimmunization with 10⁷ cells by the vaginal route elicited higher concentrations of the three CC chemokines than rectal immunization (Fig. 2b). The concentrations of each of the three CC chemokines (CCL3, CCL4 and CCL5) increased after the first and second immunizations (Fig. 3). As with the proliferative responses, the cells derived from macaques immunized with 10⁴ allogeneic PBMCs induced smaller increases in the concentrations of the three CC chemokines compared with those elicited by 10⁷ cells. The concentration of the control CCL2 showed little or no change in the alloimmunized macaques (Fig. 3). The control macaque treated with 10⁷ autologous PBMCs showed no change in the concentrations of any of the CC chemokines. Thus, as with the T-cell proliferative response, a dose-dependent increase in the concentration of the three CC chemokines was found in the alloimmunized macaques.

**Antibodies to CCR5**

Serum antibodies to CCR5 were not detected by ELISA before immunization, but all except one of the eight macaques developed CCR5 antibodies after alloimmunization (Fig. 2c). Antibody titres showed a moderate dose-dependent increase, as immunization twice with 10⁴ cells
failed in one of the two macaques, administration of 10^6 cells induced titres of 1:25 in both macaques, but titres of 1:50–1:200 were elicited with 10^7 cells (Fig. 2c). Vaginal alloimmunization was as effective as rectal alloimmunization. The macaque immunized with autologous cells failed to develop antibodies to CCR5.

SIV infectivity of CD4^+ T cells in macaques alloimmunized by the mucosal route

Analysis of SIV infectivity of PHA-stimulated CD4^+ cells was carried out before and after alloimmunization with PBMCs in three macaques; two received 10^6 and one received 10^7 cells. The results suggest a dose-dependent inhibition of infectivity in the CD4^+ T cells after alloimmunization, compared with those before alloimmunization (Fig. 4a). Thus, mucosal alloimmunization induced a decrease in SIV infectivity in CD4^+ T cells separated from the circulating PBMCs. However, whether there was a similar or indeed enhanced inhibition of infectivity of CD4^+ T cells in the mucosa or the regional lymph nodes was not ascertained.

SIV infectivity of CD4^+ T cells in multiparous macaques

Investigation of SIV infectivity of CD4^+ T cells separated from PBMCs of multiparous macaques was based on the assumption that they were alloimmunized by the semiallogeneic fetal cells. Indeed, a significant dose-dependent inhibition of SIV infectivity of CD4^+ T cells was found when compared with nulliparous macaques (Fig. 4b). The results of SIV infectivity of CD4^+ T cells were similar to those elicited by mucosal alloimmunization.

DISCUSSION

The potential for inducing alloimmunization during unprotected sexual intercourse with a monogamous partner has recently been studied and showed a significant alloimmune response to the partner’s mononuclear cells (Peters et al., 2004). The aim of this investigation was to study direct mucosal exposure to unmatched mononuclear allogeneic cells in non-human primates to find out whether alloimmunization could be elicited through the intact rectal or vaginal mucosa. T-cell proliferative responses were elicited.
in all eight macaques immunized by non-traumatic administration of alloimmune mononuclear cells into the rectum or vagina. The alloimmune responses were dose-dependent, as 10^7 PBMCs elicited significant increases in T-cell proliferation (SI > 3) in all four macaques (2/2 after vaginal and 2/2 after rectal immunization) after the first mucosal application. In contrast, only 1/4 macaques allo-vaginal and 2/2 after rectal immunization) after the first immunization. However, all eight macaques developed significant allo-immune responses after the second immunization. Thus, allogeneic mononuclear cells administered via the rectal or vaginal mucosa induced a dose-dependent secondary allo-immune response in the circulating cells. The interpretation of the data must be viewed with caution, as MHC typing of the macaques was not carried out due to lack of typing reagents for Chinese macaques. Nonetheless, all the animals were outbred and from records we were able to establish that none of the animals was related. Given the published complexity of the phenotypes in both MHC class I and II (Otting et al., 2005), it is highly unlikely that the animal chosen as the donor of the allogeneic cells would carry the same alleles as the recipients. Whilst it is unlikely that 10^6–10^7 cells are found in ejaculates of healthy males, 10^5–10^7 HLA- cells have been reported in ejaculates from subjects with sexually transmitted infections, as there is an increased number of neutrophils, macrophages, CD4^+ T cells, epithelial cells, germ cells and cell-free HLA antigen (Quayle et al., 1997).

Investigation of CC chemokines derived by mitogenic stimulation of CD8^+ -enriched cells showed that CCL3, CCL4 and CCL5, but not CCL2, were significantly increased in concentration after both the first and second alloimmunizations. The response was dose-dependent, as was found with T-cell proliferation. The combined concentration of the three CC chemokines after the second immunization reached levels between 5453 and 14 466 pg ml^{-1}, which is within the range of 2500–25 000 pg ml^{-1} required to inhibit 90–95 % of SIV replication in vitro (Wang et al., 1999b). Hence, levels of CC chemokines can be reached that may inhibit SIV replication and this might also apply to the rectal and vaginal mucosa, as high numbers of CCL5- and CCL4-secreting cells were found in T cells eluted from the rectal mucosal tissue (Lehner et al., 2000b). In addition to the CC chemokines binding CCR5 and preventing SIV transmission, CD8-SF was also significantly increased by alloimmunization and may contain other SIV inhibitory factors (Levy et al., 1996). Recently, a protease inhibitor was reported to account for the anti-HIV activity generated from CD8^+ T cells (Mackewicz et al., 2003), as well as a ribonuclease (Rugeles et al., 2003).

SIV infectivity of activated CD4^+ T cells was then evaluated in vitro before and after mucosal alloimmunization. A significant dose-dependent inhibition of SIV infectivity of CD4^+ T cells was found in alloimmunized macaques when compared with their pre-immunized cells. A similar inhibition of SIV infectivity of activated CD4^+ T cells was found in multiparous macaques compared with male macaques. This suggests that alloimmunization of multiparous macaques by the semi-alloageneic fetuses had occurred which elicited inhibition of SIV infection of CD4^+ T cells. However, the controls in this study were male macaques, as matched female nulliparous macaques were not available, it is most unlikely that the male macaques had been alloimmunized. Furthermore, we cannot be certain that the decrease in SIV infectivity in vitro will correlate with that found in vivo. Mucosal alloimmunization will now be investigated in Indian macaques, which will be challenged in vivo with SIV by the mucosal route of immunization.

In view of the antibodies elicited against CCR5 on xenoinmunization in macaques (Lehner et al., 1999) and alloimmunization in humans (Wang et al., 2002), we examined the possibility that mucosal alloimmunization might also induce antibodies to CCR5. A dose-dependent increase in the titre of IgG antibodies to CCR5 was found in the sera of alloimmunized macaques, which may enhance blocking of CCR5 by CD8^+ cell-derived CC chemokines and SF, thereby preventing SIV infection. Indeed, CCR5 antibodies enhance CC chemokine inhibition of SIV transmission (Lehner et al., 2001). Anti-CCR5 antibodies are readily raised not only in macaques, but also in humans; indeed, antibodies to CCR5 have been detected in normal immunoglobulin used for therapeutic purposes (Bouhlal et al., 2001). It is not clear why alloimmunization in contrast to autoimmunization should elicit CCR5 antibodies, but the most likely interpretation is that alloimmunization elicits cytokines and chemokines and upregulates co-stimulatory molecules, which may facilitate an immune response to CCR5 expressed on macrophages, dendritic cells and T cells. The mechanism responsible for preventing SIV or HIV infection is likely to be that of receptor dimerization (Vila-Coro et al., 2000) and/or downmodulation of the cell-surface expression of CCR5 (Amara et al., 1997; Mack et al., 1998). However, antibodies to CCR5 may also block HIV transmission by steric hindrance.

The results of mucosal alloimmunization in macaques are consistent with systemic alloimmunization in humans, in whom increased CC chemokine, CD8-SF and CCR5 antibody levels and decreased infectivity of CD4^+ T cells with HIV were found (Wang et al., 1999b, 2002). Indeed, the results of direct mucosal alloimmunization are also consistent with the alloimmune responses elicited in humans practising unprotected sexual intercourse with significant in vitro resistance to HIV-1 infection (Peters et al., 2004). Although we have not investigated directly the effect of mucosal alloimmunization on local mucosal immunity, the finding of systemic alloimmune responses and inhibition of SIV replication in circulating CD4^+ T cells argue strongly in favour of local alloimmunization. We suggest that mucosal alloimmunization by HLA antigens in seminal fluid during unprotected sex may contribute to immune protection against HIV transmission, as has been widely
reported in seronegative subjects at risk of HIV infection (Beyrer et al., 1999; Garzino-Demo et al., 1999; Goh et al., 1999; Kaul et al., 2000; Mazzoli et al., 1997; Pinto et al., 1995). This is potentially an important finding, as alloimmunization induces a powerful immune response and may be developed into a vaccination strategy to prevent or inhibit sexually transmitted HIV infection (Lehner et al., 2000a). Mucosal alloimmunization with selected alloantigens that would cover 90% of a given population needs to be explored.

ACKNOWLEDGEMENTS

This work was supported partly by grants from the National Institute of Health, Washington, DC, and Guy’s and St Thomas’ Special Trustees.

REFERENCES


