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Mucosal immunization with PLGA-microencapsulated DNA primes a SIV-specific CTL response revealed by boosting with cognate recombinant modified vaccinia virus Ankara

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Abstract

Systemically administered DNA encoding a recombinant human immunodeficiency virus (HIV) derived immunogen effectively primes a cytotoxic T lymphocyte (CTL) response in macaques. In this further pilot study we have evaluated mucosal delivery of DNA as an alternative priming strategy. Plasmid DNA, pTH.HW, encoding a multi-CTL epitope gene, was incorporated into poly(D,L-lactic-*co*-glycolic acid) microparticles of less than 10 μm in diameter. Five intrarectal immunizations failed to stimulate a circulating vaccine-specific CTL response in 2 Mamu-A*01^{+} rhesus macaques. However, 1 week after intradermal immunization with a cognate modified vaccinia virus Ankara vaccine MVA.HW, CTL responses were detected in both animals that persisted until analysis postmortem, 12 weeks after the final boost. In contrast, a weaker and less durable response was seen in an animal vaccinated with the MVA construct alone. Analysis of lymphoid tissues revealed a disseminated CTL response in peripheral and regional lymph nodes but not the spleen of both mucosally primed animals.

Keywords: Vaccine; DNA; Microparticles; MVA; HIV; Multiepitope; Macaque; CTL

Introduction

The development of an effective acquired immunodeficiency syndrome (AIDS) vaccine demands novel approaches as many of the more conventional vaccine modalities have proven unsuitable when tested in the simian immunodeficiency virus (SIV) primate model. The potent protection elicited by live-attenuated SIV, that has been shown to be durable, effective against high dose cell-free challenge, against challenge with virus infected cells, against challenge with heterologous virus, and against challenge via mucosal surfaces, is a paradigm for the development of an effective AIDS vaccine (reviewed by Johnson and Desrosiers, 1998). The correlates of protective immunity are still incompletely understood in this system; however, generation of a SIV-specific cytotoxic T lymphocyte (CTL) response is believed to be an important factor in control of virus load (Jin et al., 1999; Schmitz et al., 1999) and the level of CTL activity has been correlated with protective efficacy after vaccination with live-attenuated vaccine (Johnson et al., 1999) and following vaccination with a recombinant vaccinia virus vector expressing SIV *nef* (Gallimore et al., 1995). The ability of live viruses to stimulate a widely disseminated specific immune response is almost certainly a factor in their efficacy. It is likely that the most effective AIDS vaccine will stimulate both local and systemic immunity as is seen with live-attenuated SIV. Replication of the live-attenuated vaccine in regional lymphoid tissue presumably accounts for the generation of local immunity and persistence of the virus, albeit at extremely low levels, at least as far as detection in the peripheral

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circulation is concerned, is thought to be a factor in the longevity of the protective response generated. Unfortunately, live-attenuated vaccines present an unacceptable level of risk due to reversion to virulence (Whatmore et al., 1995) and an incomplete knowledge of host and viral virulence factors (Baba et al., 1995). Thus alternative, safer modalities that can stimulate qualitatively and quantitatively similar responses to those generated by live-attenuated virus are being sought.

DNA vaccination in some ways emulates infection with a live-attenuated virus. Although DNA does not replicate, it may persist for some time at the site of injection (Hanke et al., 2002) and influence the dissemination and longevity of the immune response generated. In human immunodeficiency syndrome (HIV) and SIV infection the gut is a principle portal of virus entry and regardless of route of infection lymphoid tissue associated with the gut (GALT) is a primary site of virus replication early after exposure (Veasey et al., 1998; Canto-Nogues et al., 2001). It is therefore logical to target GALT for the stimulation of protective immune responses against HIV and SIV. There is at present relatively little information about DNA immunization in the gut. Direct administration of measles virus hemagglutinin (HA)-encoding plasmid DNA to mucosal surfaces has been shown to induce measles virus-specific CTL in mice; however, the oral route of administration was inefficient without the use of mucosal adjuvants (Etchart et al., 1997). Microparticles composed of poly(D,L-lactic-*co*-glycolic acid) (PLGA), which is biocompatible and has an extensive record of safe use in humans, have been shown to efficiently deliver protein antigens following gut administration eliciting both systemic and mucosal immune responses (Eldridge et al., 1991). This is believed to be due to the fact that microparticles of less than 10 μm are readily taken up by macrophages and intestinal M cells leading to antigen presentation in regional inductive immune sites. More recently, it was shown that PLGA-encapsulated plasmid DNA elicits systemic and mucosal antibodies to the encoded antigen after oral delivery in mice (Jones et al., 1997). In the human rotavirus murine-challenge model protective immune responses have been generated after oral gavage with microencapsulated DNA encoding rotavirus VP6 (Chen et al., 1998) and VP4 and VP7 (Hermann et al., 1999). Furthermore, the induction of systemic and local antibodies and CTL responses has been demonstrated in mice immunized orally with microparticles containing DNA encoding HIV Env glycoprotein (Kaneko et al., 2000).

One particularly promising approach for the stimulation of strong and durable CTL responses is the DNA prime–viral vector boost regimen (reviewed by Seder and Hill, 2000). A synthetic multiepitope gene (Hanke et al., 1998) encoding human immunodeficiency virus type 1 (HIV-1) CTL epitopes and including the SIV $Gag_{181–189}$ CTL epitope that is restricted by the Mamu-A*01 rhesus macaque major histocompatibility complex (MHC) class I molecule (Miller et al., 1991; Allen et al., 1998) has been shown to prime a $CD8^+$ CTL response in macaques following intradermal administration with a gene gun. Circulating $CD8^+$ CTL were demonstrated following boosting with modified vaccinia virus Ankara (MVA) expressing the same multiepitope gene (Hanke et al., 1999).

In the small-scale pilot study reported here, we have extended the previous study to determine whether mucosally administered HIV multiepitope DNA induces directly or primes a CTL response. PLGA microparticles containing plasmid DNA were formulated using an optimized procedure utilizing pharmaceutically approved reagents (Tinsley-Bown et al., 2000). The particles were made to an optimal size to facilitate their uptake by M cells of the gut and were administered rectally.

Results and discussion

Three batches of microparticles containing the multiepitope plasmid DNA pTH.HW were made. Analysis of DNA incorporation per milligram PLGA showed that 5.95 μg of DNA was incorporated in batch 1, 8.5 μg of DNA was incorporated in batch 2, and 8.4 μg of DNA was incorporated in batch 3. In all batches, greater than 90% of the particles were found to be less than 10 μm in size with an average size of 2.7 μm and therefore of a size suitable for uptake through M cells. The external structure of particles was examined by electron microscopy and generally the particles were found to be spherical with smooth surfaces. Next, the physical state of the encapsulated DNA was examined following release into solution. Gel electrophoresis revealed a high proportion of supercoiled form. Transfection of 293 cells with extracted DNA showed that biological activity was present (data not shown). To determine if microparticles could be phagocytosed and instigate expression of the encapsulated DNA within the cells, microparticles were added to monolayers of the mouse monocyte macrophage cell line RAW 264.7. Following incubation for 72 h, expression of the HW immunogen was shown by detection of the SV5 Pk tag epitope by immunofluorescent staining with monoclonal antibody on fixed permeabilized cells (Fig. 1). Expression was seen in the majority of cells incubated with between 5 and 20 μg of DNA.

To determine the immunogenicity of the microencapsulated DNA, two rhesus macaques (Z14, Z55) positive for the Mamu-A*01 MHC were immunized intrarectally with microparticles (batch 1) containing 300 μg pTH.HW at weeks 0, 4, and 8. Significant lytic activity was not detected at any effector-to-target ratio (E:T) in the range 6:1 to 200:1 in either animal in peptide-restimulated peripheral blood mononuclear cells (PBMC) taken 3 weeks after the first immunization, 3 weeks after the second immunization, or 3 weeks after the third immunization (Fig. 2). This result was no different than that obtained in macaques vaccinated with the same plasmid DNA construct administered intradermally with the Dermal XR particle delivery device (Pow-

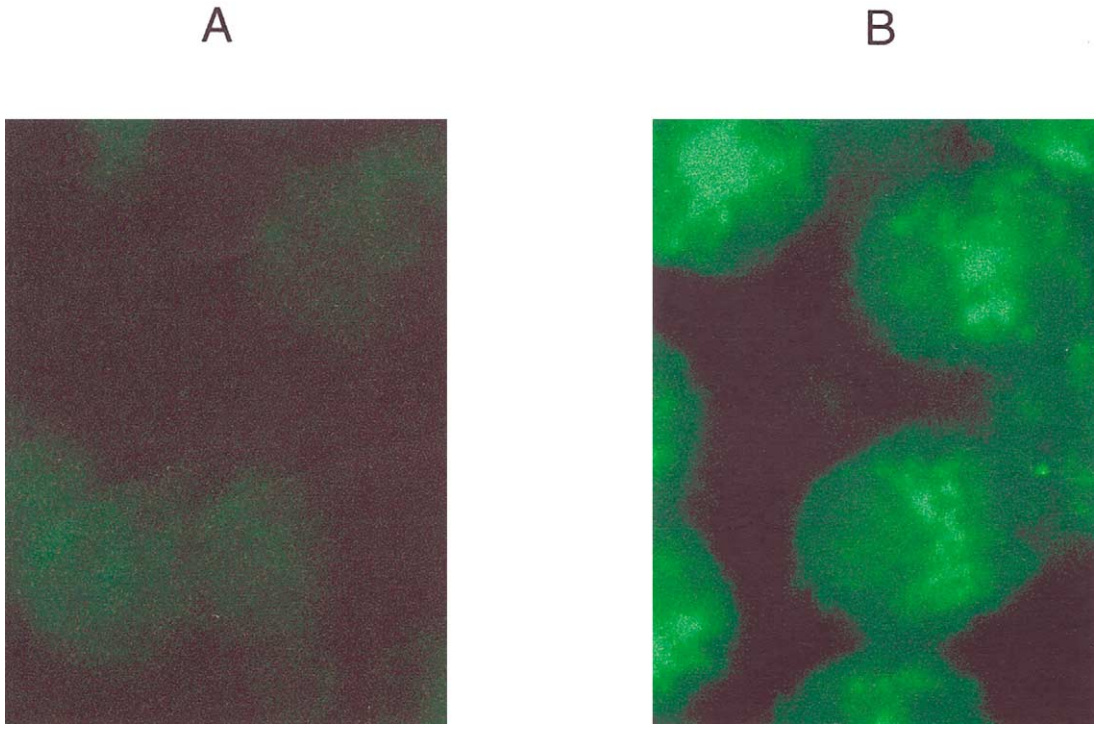

Fig. 1. RAW 264.7 cells phagocytose PLGA/pTH.HW microparticles and express the HW immunogen. Untreated cells (A) and cells exposed to microencapsulated DNA (B) were fixed and permeabilized and recombinant protein was detected by staining with a monoclonal antibody with specificity for the SV5 Pk epitope followed by FITC-conjugated anti-mouse lg goat $F(ab')_2$.

derJect Vaccines Inc.) (Hanke et al., 1999). Oral administration of the microparticles to BALB/c mice at a dose of 200 μg also failed to stimulate significant CTL activity to the H-2D^d-restricted HIV-1 epitope RGPGRAFVTI, encoded by the multiepitope construct, in splenocytes or in mononuclear cells isolated from mesenteric lymph nodes 3 and 9 weeks after immunization (data not shown). To determine if a higher dose of DNA would stimulate a directly measurable systemic CTL response, animals Z14 and Z55 next received two further doses of 3 mg DNA in microparticles (a 50:50 mix of batches 2 and 3) at weeks 35 and 39. Again no significant lytic activity was detected in peptide-restimulated PBMC taken 2 weeks after each of these subsequent immunizations. An equivalent dose could not be tested in mice due to the limitation imposed by volume. To determine if the macaques had been primed for a CTL response, both animals were further immunized intradermally, 9 weeks after the final DNA immunization, with the cognate recombinant MVA.HW expressing the polyepitope gene. A naive Mamu-A*01$^+$ macaque (445) was also similarly immunized. On the day of immunization, lytic activity was not detected in either the DNA vaccinated or the naive animal (Fig. 2). When tested 1 week after immunization with the MVA construct, all three animals revealed $Gag_{181–189}$-specific CTL activity in restimulated PBMC; however, in the non-DNA immunized animal this response was weak, only reaching above the 10% cut off at effector-to-target ratios of 50:1 and greater and was only 14% above cutoff at 200:1. Furthermore, 1 week later, lytic activity was undetectable in this animal and remained undetectable subsequently, 4, 6, and 8 weeks after MVA vaccination even at an E:T of 200:1. In contrast, 1 week after MVA immunization, significant lytic activity of 16 and 21% was seen in animals Z14 and Z55, respectively, even at the lowest E:T tested of 6:1 rising to 28 and 43% at 200:1. Furthermore, the responses were persistent (Fig. 2) with between 20 and 14% lytic activity detected 4 weeks after MVA vaccination even at 12.5:1 and up to 31% killing detected at higher E:T. To determine if the responses could be further boosted, animals were immunized again with MVA.HW. One week later, all animals had higher levels of lytic activity. In 445, lytic activity returned to a level greater than seen after the first MVA immunization, although significant killing was undetectable below an E:T of 25:1 and the response was not durable. In contrast, Z14 and Z55 had persistent responses (Fig. 2) and although these had declined somewhat by 12 weeks after the boost, killing was still detectable above the cutoff at an E:T of 12.5:1.

The accelerated response in DNA-immunized macaques following MVA immunization is similar to that reported previously in macaques vaccinated using the gene-gun followed by MVA (Hanke et al., 1999); indeed in that study, only one of three macaques had lytic activity by 1 week after the first MVA vaccination but all animals developed CTL responses subsequently. Although only one Mamu-A*01$^+$ macaque was available to us in this study as an

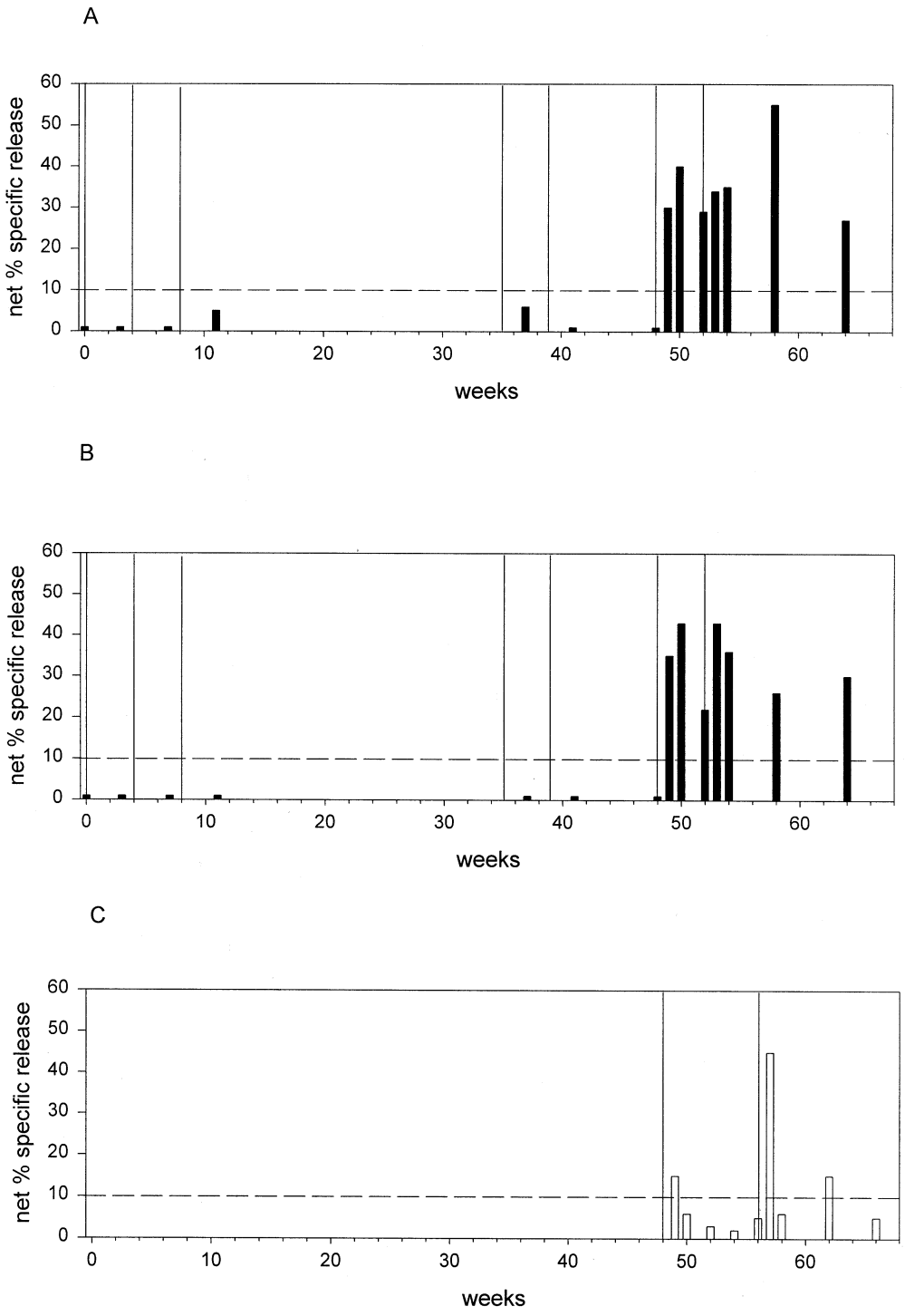

Fig. 2. Longitudinal analysis of CTL activity in SIV $Gag_{181–189}$ peptide-restimulated PBMC taken from vaccinated macaques. Macaques Z14 (A) and Z55 (B) were vaccinated first with 300 μg three times followed by 3 mg doses given twice of plasmid DNA pTH.HW encapsulated in PLGA intrarectally. Subsequently, Z14 and Z55 together with a non-DNA-primed macaque, 445 (C), received two intradermal immunizations with 5×10^8 PFU of MVA.HW as indicated by the vertical divisions. Results are shown as net percentage specific lysis following subtraction of lysis obtained on peptide unpulsed autologous B-LCL for an effector:target ratio of 100:1.

MVA-alone control; the results from this animal were completely consistent with those obtained from the two MVA-alone controls used in the previous study (Hanke et al., 1999). In that study a single vaccination with MVA failed to stimulate SIV $Gag_{181–189}$-specific $CD8^+$ cells detectable with MHC-peptide tetrameric complexes and even two immunizations induced only a slow and very weak response. In the present study, lytic activity was detected after re-

stimulation but this was weak after a single immunization and transient even after two immunizations. Taken together, these results indicate that prior exposure to cognate DNA primed the CTL response and that mucosal exposure to DNA could effectively prime a circulating response.

Next the distribution of CTL activity was analyzed postmortem in a variety of lymphoid organs. CTL activity was detected in iliac, inguinal, mesenteric, and axillary lymph nodes but not in the spleen of both mucosally primed animals (Fig. 3). In the animal vaccinated with MVA without DNA priming, a titratable CTL response was detected only in cells from axillary lymph nodes at and above an E:T of 50:1 and killing reached a level of only 28% even at the highest E:T of 200:1. Cells from all other tissues tested were essentially unreactive, or had low levels of lytic activity that did not titrate in a reproducible manner. Interestingly, both animals that had been vaccinated first with DNA had strong CTL memory responses in the interior iliac lymph nodes. This is particularly encouraging since these nodes drain the rectal–genital tract, are known to be an early site of virus replication following mucosal challenge (Spira et al., 1996), contain precursor CTL following infection of macaques systemically with attenuated SIV (Cranage et al., 1997) and immunization targeting these nodes has been associated with protection from virus challenge (Lehner et al., 1996). The failure to detect CTL activity in splenocytes was surprising and may indicate that the response detected in PBMC represents cells that are recirculating to local tissues rather than resident in the secondary lymphoid compartment. Lytic activity was not detected in direct, unstimulated CTL assays using cells from any of the lymphoid tissues including blood and intraepithelial lymphocytes recovered from jejunum. Cells from jejunal lamina propria had poor viability and bacterial contamination was shown to be present. However, in macaque Z14 we were able to obtain cells from tonsils and these revealed specific lytic activity after restimulation, suggesting that the DNA-prime MVA boost regimen may have stimulated a disseminated mucosal immunity.

Further work is required to determine the optimal requirements for mucosal priming with DNA. In this pilot study, relatively large doses of DNA were administered and it was not possible to define whether high and repeated doses were necessary. It is unlikely that the current formulation is optimal. While incorporation of the DNA into the particle has the advantage of facilitating control of the release profile, such encapsulation may also physically damage the DNA. An alternative procedure has been described recently where DNA adsorbed onto cationic PLGA microparticles given parenterally to small animals and primates elicited enhanced HIV-specific immune responses compared to those obtained after naked DNA immunization (O'Hagan et al., 2001). Furthermore, in mice, enhanced mucosal and systemic immunity was seen following intranasal administration (Singh et al., 2002). For optimization studies, it will be important to use quantitative assays such as staining with tetrameric MHC–peptide complexes. A faulty batch of tetramers precluded such analysis in the present study.

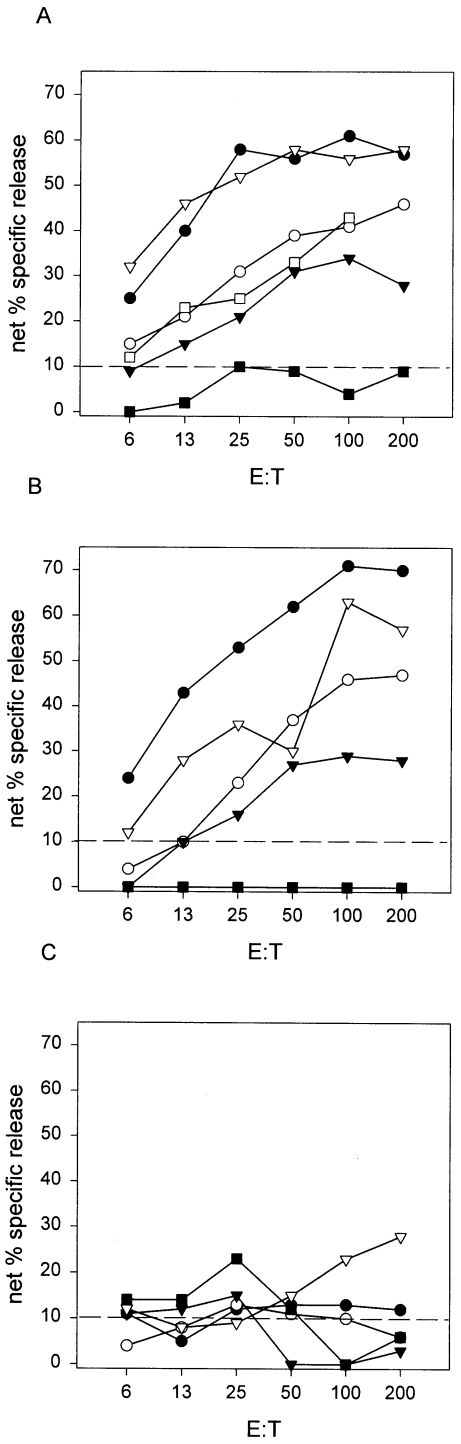

Fig. 3. CTL activity in cells recovered from lymphoid compartments of immunized macaques *postmortem*. Net percentage specific lysis is shown for a range of effector:target using SIV $Gag_{181–189}$ peptide-restimulated cells recovered from (●) iliac, (○) inguinal, (▼) mesenteric, and (▽) axillary lymph nodes, (■) spleen, and (□) tonsil of macaques primed with pTH.HW DNA and boosted with MVA.HW (A and B: macaques Z14 and Z55, respectively) and an animal immunized only with MVA.HW (C: macaque 445).

The mechanism by which microparticles deliver DNA is poorly understood. Although microparticles may directly facilitate the uptake of DNA into antigen-presenting cells and may prolong gene expression (Singh et al., 2002), they may also have an additional adjuvanting property since empty particles coadministered with DNA into the peritoneal cavity of mice have been shown to enhance the humoral immune response to the encoded antigen (Fooks et al., 2000). Clearly, further studies are required to establish, in primates, whether the PLGA is essential for the priming activity of the mucosally delivered DNA.

The rectum is an immune inductive site (Lehner et al., 1994; Klavinskis et al., 1996); however, for clinical use, oral or nasal administration of microparticles may be more acceptable. For oral administration additional encapsulation to allow passage through the acidic environment of the stomach would be necessary. Delivery of DNA by these routes is likely to be relatively inefficient compared to the use of specific vectors. For example, we have recently demonstrated that oral dosing of mice with replication-deficient recombinant adenovirus expressing the measles virus nucleocapsid protein was much more efficient than oral administration of PLGA microencapsulated plasmid DNA encoding the same antigen (Fooks et al., 2000; Sharpe et al., 2002). Nonetheless, the advantages of avoiding an antivector immune response may outweigh the increased efficiency of vector delivery. Having demonstrated, in this pilot study, the feasibility of priming a CTL response by mucosal delivery of DNA in primates, it will now be important to define the optimal combination of mucosal and systemic delivery modalities to elicit protective immune responses.

Materials and methods

Plasmid DNA and microencapsulation

The plasmid, designated pTH.HW, encoding multiple HIV-1 CTL epitopes and several SIV epitopes together with the SV5 Pk tag epitope was essentially as described by Hanke et al. (1998), but the Mamu-A*01-restricted SIV Gag epitope encoding sequence was modified to encode the optimal epitope $Gag_{181–189}$ as described by Allen et al. (1998). The plasmid was prepared using standard methods and purified to be pyrogen-free using the EndoFree Plasmid Maxi Kit (Qiagen).

DNA was microencapsulated in PLGA as has been described previously (Tinsley-Bown et al., 2000). Briefly, a solution of two PLGA polymers RG502 and RG503 (Boehringer Ingleheim, Germany) were mixed 50:50 in ethyl acetate (4 ml) and emulsified with 0.4 ml of a 10 mg/ml solution pTH.HW in STE buffer (100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA pH 8.0) using a Waring blender (PGC, USA) set to low power (18,000 rpm). Four 15s on-cycles punctuated with 15s rest periods were used to facilitate emulsification. This emulsion was added immediately to an 8% aqueous polyvinyl alcohol solution (66 ml) preheated to 30°C and emulsified using the low power setting of the blender. Again a schedule of 15s on-cycles punctuated with a 15s rest period was used. The resting double emulsion was dispersed rapidly in distilled water, preheated to 37°C with stirring, and stirred at room temperature for 1 h. The microparticles were recovered by centrifugation (18,000 *g* for 25 min), washed four times with distilled water, and lyophilized. Particles were stored at −20°C with desiccant prior to use.

Size distribution analysis over a 1 to 30-μm range was carried out using a Microbial Systems Cellfacts analyzer fitted with a 60-μm orifice. The structure of the microparticles was analyzed by scanning electron microscopy (SEM). The external structure of the freeze-dried particles was determined by gently applying the microparticles to adhesive carbon tabs mounted on SEM specimen stubs. The specimen stubs were coated with approximately 5 nm gold by ion beam evaporation prior to examination in a Philips XL30FEG scanning electron microscope operated at 2 kV accelerating voltage.

To determine the quantity of plasmid DNA incorporated into PLGA microparticles, aliquots (10–15 mg) were vortexed with 200 mM NaOH in a 1 ml volume and incubated at 120°C for 10 min. The aqueous phase was recovered by centrifugation at 8500 *g* for 10 min in a microcentrifuge and the DNA concentration was determined by UV absorption at 260 nm against 200 mM NaOH.

Plasmid DNA was extracted for biophysical analysis by vortexing aliquots of microparticles (20 mg) with chloroform (0.5 ml) and incubating at room temperature for 30 min. STE buffer (1 ml) was added and the mixture was vortexed to facilitate phase separation. The mixture was centrifuged for 10 min at 8500 *g* in a microcentrifuge and the aqueous phase containing the DNA was removed. The concentration of DNA was determined by UV spectroscopy. The recovered DNA was analyzed by electrophoresis of 0.1 μg aliquots on 0.8% agarose in TBE gels. Adequate separation of the bands for supercoiled, open circular, and linear DNA was obtained by running the gel for 1 h at 100 V, 400 mA.

Biological activity of recovered DNA was assayed by transfection of 293 cells. Monolayers (~80% confluent) of 293 cells in 6-cm^2 dishes were transfected with recovered DNA using the Effectine Transfection reagent (Qiagen). One hundred and fifty microliters of EC buffer was added to 1 μg of recovered DNA. Eight microliters of enhancer was added and incubated for 5 min at room temperature. Following centrifugation (10 s, microcentrifuge), 25 μl of Effectine reagent was added and mixed by pipetting five times. One milliliter of warm growth medium (Dulbecco's MEM + 10% FCS) was added to the transfection mix and the mixture was added dropwise, immediately to a washed monolayer of 293 cells in 4 ml of growth medium. Monolayers were incubated at 37°C in a mixture of 5% CO_2 +

95% air for 12 h. Expression from the transfected DNA was detected by immunostaining using a mouse anti-Pk-tag monoclonal antibody (Serotec). After washing to remove residual serum and debris, monolayers were fixed with 4 ml cold methanol for 10 min. After further washing, cells were blocked with phosphate-buffered saline (PBS) containing 10% FCS for 60 min and then stained with monoclonal antibody diluted 1/2000 in blocking buffer. After 60 min monolayers were washed and bound antibody was detected by reaction with rabbit anti-mouse immunoglobulin–horseradish peroxidase (HRP) conjugate (Dako) diluted 1/2000. After a further 60 min monolayers were washed extensively in PBS and HRP-labeled cells were detected by reaction with H_2O_2 and AEC.

Expression in vitro directly from phagocytosed microparticles was investigated on monolayers of RAW 264.7 cells (European Collection of Animal Cell Cultures No. 91062702). Multichamber microscope slides were seeded with cell suspensions in DMEM containing 10% FCS + 0.1 mg/ml kanamycin to give approximately 80% confluency after overnight incubation. Microparticles suspended in DMEM + 0.1 mg/ml kanamycin were added to wells to give a range of DNA concentrations between 5 and 20 μg DNA per well. Negative control wells were left untreated. After 3 h at 37°C, cell sheets were washed three times with PBS to remove excess microparticles and fresh growth medium was added. Following incubation for 72 h, cell monolayers were washed once in PBS and fixed using 0.5% paraformaldehyde for 10 min at room temperature. Cells were permeabilized by treatment with 50% ethanol for 5 min, then 70% ethanol for 10 min, followed by 50% ethanol for 3 min. After washing three times with PBS, nonspecific reactive sites were blocked by incubation with 2% FCS in PBS for 1 h at 4°C. Fixed, permeabilized cells were then incubated overnight at 4°C with 1/100 dilution of mouse monoclonal antibody anti-SV5 Pk tag (Serotec). Bound antibody was detected after a further wash in PBS by incubation for 1 h at room temperature with 1/100 dilution of FITC-conjugated goat $F(ab')_2$ anti-mouse lg (Sigma). Washed cells were viewed using a UV microscope.

Animals and immunization

Rhesus macaques used in this study were bred within the United Kingdom and housed according to the Home Office Code of Practice (1989). Animals were anesthetized by intramuscular injection with ketamine hydrochloride (Vetalar, Park Davis Pontypool, UK) for all procedures requiring removal from their cages including femoral venipuncture and immunization. Animals with the Mamu-A*01 tissue type were selected for this study by sequence-specific primer (SSP)-PCR and direct sequencing (Knapp et al., 1997).

Macaques were immunized with 3 ml doses of PLGA microparticles delivered to the rectal mucosa without trauma using a soft polypropylene 6FG urinary catheter that was inserted approximately 8 cm. Subsequently, animals were immunized by intradermal injection of 5×10^8 plaque forming units (PFU) of MVA.HW expressing the HIV-1 multiepitope in 300 μl sterile PBS in multiple sites in the thigh.

CTL assay

PBMC were isolated by Ficoll–Paque (Pharmacia, Milton Keynes, UK) density centrifugation from heparinized blood and washed in medium consisting of RPMI 1640 (Gibco, Paisley, UK) supplemented with L-glutamine (2 mM) (Gibco), penicillin (50 U/ml)/streptomycin (50 μg/ml) (Gibco), and 10% heat-inactivated fetal bovine serum (Flow, Oxon, UK). Splenic mononuclear cells (MNC) and lymph node MNC were obtained by teasing these tissues and washing cells in culture medium. Splenic MNC were purified on Ficoll–Paque cushions. Intraepithelial lymphocytes and lamina propria lymphocytes were obtained from jejunum by mechanical and enzymatic dissociation as described previously (Polyanskaya et al., 2001). Cells were cultured in RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (50 U/ml)/streptomycin (50 μg/ml), 2-mercaptoethanol (0.05 mM), HEPES buffer (25 μM), and 10% heat-inactivated fetal bovine serum containing $Gag_{181–189}$ peptide at 20 μg/ml. Lymphocult T was added to a final concentration of 10 IU/ml on days 3 and 7. Precursor CTL were restimulated in vitro on day 7 by the addition of mitomycin C (25 μg/ml, 1 h)-inactivated, peptide-pulsed autologous B-lymphoblastoid cells (B-LCL). At day 13, effector cells were washed three times and used in triplicate at various effector-to-target ratios in a standard chromium release assay. Autologous B-LCL targets were pulsed with $Gag_{181–189}$ peptide and labeled with 150 μCi ^{51}Cr for 2 h. After washing three times, 5000 cells were added to each test well. Chromium release was determined in the supernatants after 4 h. Spontaneous release and total release were determined from wells containing target cells and medium alone or with 5% Triton X-100. Responses were considered positive if there was at least 10% specific lysis above that obtained from control targets. Spontaneous release from target cells was less than 18%.

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