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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The initial proposal was to produce multiple antigens; to use oxidised mannan as a delivery system and finally to see the effect of single and multiple other antigens in experimental tumours in mice. A number of the antigens have been made including: a) MUC1 – glycosylated; full length MUC1 lacking VNTR; mutant forms of MUC1; b) nm23; c) Claudin 7 peptide epitopes synthesised; d) we are obtaining the Her2/neu protein.

Secondly, we have expanded the delivery system to not only include mannan, but also include particles capable of targeting dendritic cells.

Finally, several tumor models are currently in use:- a) Tumor models are MUC1 transgenic models; b) mouse tumours expressing the antigens and c) MCSF-7-ras growing in SCID mice which will adoptively receive cells; d) comparison of the mannan – DC targeting vectors and DNA will be done to conclude this project.

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INTRODUCTION

The therapy of breast cancer is largely static. Thus surgery, radiotherapy, cytotoxic drugs and hormones have almost reached the limit of their use, although Taxol is now making some impression. However, it is appropriate that new immunotherapeutic approaches be examined, and in this light, we have already conducted a number of clinical trials of mannan MUC1 in patients. In these patients we see reasonable immune responses frequently with antibody produced, some cellular responses, but to date no major tumour shrinkage has been seen. Indeed, other than for melanoma, it is a rare event to see solid tumors disappear. We are therefore re-examining all aspects of immunotherapy and in particular to : -

1) Examine the immunotherapeutic role of a number of antigens including nm23, p53, Her2/neu, pim-1 (which is present in breast cancer), Cripto-1, and more recently, fully glycosylated <u>whole MUC1</u>, MUC1 less the VNTR. MUC1 mimics and mutants and Claudin 7.

2) Modes of immunisation/delivery. We are concentrating on oxidised mannan as a delivery system as it is so potent in mice for T1 type cellular responses, but now are also examining other methods of delivery which target dendritic cells. This will be compared with a DNA delivery system.

3) Improved assay methods for cellular immunity and this includes the ELISPOT assay to measure the frequency and cell type source of critical cytokines from antigen-specific reactive T cells.

4) These strategies have been assembled together into immunotherapeutic studies, which are in progress, using inbred mice and mouse tumours; tumours expressing a human transgene (MUC1) and MCF-7-ras (HLA-A2^{+}) growing in SCID mice which will adoptively receive cells from immunised HLA-A2^{+} mice.

BODY

Statement of work from application

Vaccine development against novel breast cancer antigens

Task 1 **Produce recombinant proteins Her2/neu, nm23, p53, Bcl2, bax and other antigens for linkage to mannan (months 1-18).**

- Clone cDNAs for Her2/neu, nm23, p53, Bcl2 and bax into the pGEX expression vector (months 1-12).
- Produce small amounts of protein for *in vitro* characterisation of antigens (months 6-14).
- Conjugate the recombinant proteins to mannan for *in vitro* characterisation (months 12-18).
- Examine other antigens: Cathespin D, Brca1/2, EGFr, Cripto-1, and amphiregulin.

Products/deliverables: Pure recombinant fusion proteins (Her2/neu, nm23, p53, Bcl2, bax and others) and their mannan conjugates.

Task 2 **Study the *in vivo* immune response of mannan-conjugated and non-mannosylated free antigens in mice (months 18-24).**

- Immunise C57BL/6, BALB/c and HLA-A2/K^b transgenic mice with immunogens and study antibody response, CTL and proliferation to the antigens (months 16-24).
- Immunise mice with HLA-A2.1 peptides of Her2/neu and p53 conjugated to KLH and mannan and study the immune responses(months 18-24).

Products/deliverables: Her2/neu, p53 peptide-KLH-mannan conjugates.

Task 3 **Study tumor protection in mice immunised with the mannan conjugates (months 24-36).**

- Immunise C57BL/6 and BALB/c mice with the various conjugates and challenge with murine tumors transfected with the various antigens (months 20-26).
- Immunise HLA-A2/K^b transgenic mice for generating lymphocytes for adoptive transfer into *scid* mice with human HLA2$^+$ breast tumors (months 24-36).
- Study the effects of T1 cytokines(γIFN, TNFα, IL-2 and IL-12) given simultaneously with mannan conjugates on tumor challenge (months 26-32).
- Develop new peptides based on known HLA-A2.1 epitopes of Her2/neu and p53 using molecular modelling and study immune responses in HLA-A2/K^b mice (months 30-36).

Products/deliverables: peptide mimics for known HLA-A2.1 epitopes of Her2/neu and p53 and their KLH-Mannan conjugates.

Task 1: **Produce recombinant proteins Her2/neu, nm23, p53, Bcl2, bax and other antigens for linkage to mannan**

These studies are progressing satisfactorily, in particular nm23 is now in production and small amounts of protein made – the same is true for p53 and Her2/neu. At this time we will go ahead with the production and testing of these prior to starting Bcl2, bax and others. An additional protein has been added – glycosylated whole MUC1. Previous studies from our Institute and elsewhere have used peptides in the MUC1 VNTR. The VNTR is a 20 amino acid repeat, which contains highly immunogenic peptides. However, on analysing the data from the patients who received mannan VNTR it was clear that large amounts of antibody could be made to the peptides, but not to glycosylated MUC1 nor to the patients' own tumour. We thought that tolerance was being broken, however MUC1 peptides do not occur in isolation, they are heavily glycosylated and therefore it is sensible to immunise with glycosylated peptides. To do this we are negotiating with Dr Henrik Clausen in Copenhagen, Denmark to add Gal NAc to synthetic VNTR. While the VNTR does contain epitopes for Class I presentation and recognition by $CD8^+$ cytotoxic lymphocytes there are also epitopes in the other region of MUC1 which we have recently identified (Pietersz et al manuscript) and we therefore have considered it more useful to use the whole extracellular MUC1 to provide more epitopes. In a second approach we are in the midst of cloning whole extracellular MUC1 sequence (see below) into the appropriate vector so it will be expressed in the human embryonic 293 kidney cells – in that way the secreted material will be glycosylated, but not recognised by human natural antibodies (anti-Gal) that react with glycosylated proteins from most animal cells. Several other antigens are on the list; pim-1 and Crypto-1 have been expressed and these will be available for use.

In a recent study using serial analysis of gene expression (SAGE) and DNA arrays, in addition to MUC1, Claudin-7 was shown to be increased >100 fold in primary breast tumours. This is the first time that the association of Claudin-7 with breast cancer was identified and it is another antigen which will be used as a target for developing CTL for breast cancer.

In short, most of the antigens are on track for production, with Bcl-2 and bax delayed pending results from the others, but substituted with whole MUC1 and Claudin-7 and pim-1.

Products/Deliverables:

Recombinant fusion proteins from a number of antigens have already been made (Her2/neu, nm23, p53, pim-1, Cripto-1) – they will soon be tested for their ability to be mannosylated and be appropriate for testing in the next part of the study. Whole human MUC1 should be expressed within the next 3 months and they rapidly determined.

Task 2: **Study the *in vivo* immune response of mannan-conjugated and non-mannosylated free antigens in mice**

Studies have commenced injecting mice with nm23 and whole MUC1 as HMFG and studies will shortly commence with the other antigens. However, several points should be made here.

1) Mannosylation vs other methods of immunisation. While the original program used oxidised mannan, analysis of our clinical results gave a disappointing lack of responsiveness of tumours. Admittedly the patients had advanced disease, but they did make antibody and CTL responses – albeit the latter were fairly weak. We were concerned that the mode of immunisation while superb in mice was not appropriate in humans and we are moving in 2 directions: -

   a) Associate Professor Magda Plebanski in our group has defined a new method of immunisation using antigens conjugated to beads. Using the beads, appropriate immune responses have developed in mice as antibodies, CTLs, but also particularly successful ELISPOT assays have been undertaken. The data for this is shown in Appendix 1.

   b) Prime boost immunisation strategy. The mice are primed by whatever method and route is under study, e.g. mannosylated protein, DNA, peptide and so on. The trick is then to follow the priming with a boost using a live vector such as vaccinia, carrying the antigen of interest. These studies were conducted and the data is shown in Appendix 2. It was of interest that the prime boost strategy for MUC1 was not efficient, but it will be applied to the other antigens

2) Measurement of immune responses. While our initial proposal planned to measure antibodies and cellular responses by way of proliferation and CTLs, we have developed a far more suitable method of measuring cellular responses: ELISPOT assays detecting γIFN production by either CD4 or CD8 cells. These tests can be done immediately, rather than after days of *in vitro* culture, and give an answer on the status of total cellular immunity, as opposed to CTLs which often takes some weeks to obtain an answer, and are limited to one effector mechanism. Typical ELISPOT data are included in Appendices 1 and 2.

Task 3: **Study tumour protection in mice immunised with the mannan conjugates.**

Over the last year the antigens have been prepared, tested and this is almost complete. The program is on track and will involve the following:

- Immunise C57BL/6 and BALB/c mice with the various conjugates and challenge with murine tumors transfected with the various antigens (months 20-26).
- Immunise HLA-A2/K^b transgenic mice for generating lymphocytes for adoptive transfer into *scid* mice with human $HLA2^+$ breast tumors (months 24-36).
- Study the effects of T1 cytokines(γIFN, TNFα, IL-2 and IL-12) given simultaneously with mannan conjugates on tumor challenge (months 26-32).
- Develop new peptides based on known HLA-A2.1 epitopes of Her2/neu and p53 using molecular modelling and study immune responses in HLA-A2/K^b mice (months 30-36).

KEY RESEARCH ACCOMPLISHMENTS

A number of accomplishments have been:

1. Preparation of antigens. Pim-1, Cripto antigens have been produced and monoclonal antibodies made to these to confirm their expression, analyse their purity and productivity. It is of interest that the antibodies to these two antigens inhibit cell growth *in vitro* and are currently now being studied *in vivo* for their growth inhibitory effects – similar to that obtained with Herceptin antibody (see enclosed results – Appendix I).

2. Modes of immunisation. The oxidised mannan work has continued, but in addition, single mannose residues have been prepared and conjugated to cells; this appears as satisfactory as the oxidised mannan. Secondly, dendritic cells have been loaded up directly with oxidised/reduced mannan and the effects noted – this is particularly relevant as the mannose receptor expressed in moderate amounts on immature dendritic cells and very large amounts on matured dendritic cells. Of further relevance is a separate study being conducted at the Austin Research Institute where human dendritic cells are targeted with mannose MUC1 for therapy in patients.

   Several of the antigens have been produced:

   a) nm23 – when this uses the DC Tag method it produces extremely high doses of antibody up to two injections (titers >1:1,000,000) and leads to CD8+ γIFN secretion. The expression of nm23 is now being tested in the tumour models to use an appropriate model for this *in vivo*.

   b) MUC1. The MUC1 antigen is produced in contrast to synthetic peptides of the repeat VNTR which are non-glycosylated we have made, with some difficulty, fully glycosylated whole molecules. This is expressed in human 293 cells and should be appropriate to use in human studies as it is more human like than any other MUC1 antigen (other than HFMG). Several variants of this have been produced including MUC1 lacking the VNTR (as the VNTR response may dominant the response entirely); MUC1 mimic peptides will be used together with the previous antigens and mutants of MUC1 have been designed based on the crystal structure of MUC1 solved recently by Dr Vasso Apostolopoulos.

   c) P53 – has been produced in Ecoli and is currently being tested and the Her2/neu protein is being provided to us by Dr Anand Gautum of Pharmexa A/S in Denmark so we do not have to make this.

3. In vivo models are now established and include: a) mouse tumours (of BALB/c origin) to be grown in BALB/c mice. Some of them express the Pim-1 and Cripto antigens (which cross react with humans) and small amounts of nm23 and can be used in mouse studies. Secondly for the MUC1 studies, P815 expressing MUC1 can be used – while this is ultimately rejected, early rejection or failure to grow is a measure of immungenecity. Finally, the MCSF-7-ras tumour is obtained from France – this grows in SCID mice which will be the recipient of cells from HLA-A2 mice immunised with the various agents.

REPORTABLE OUTCOMES

The following manuscripts have been submitted for review or are being prepared:

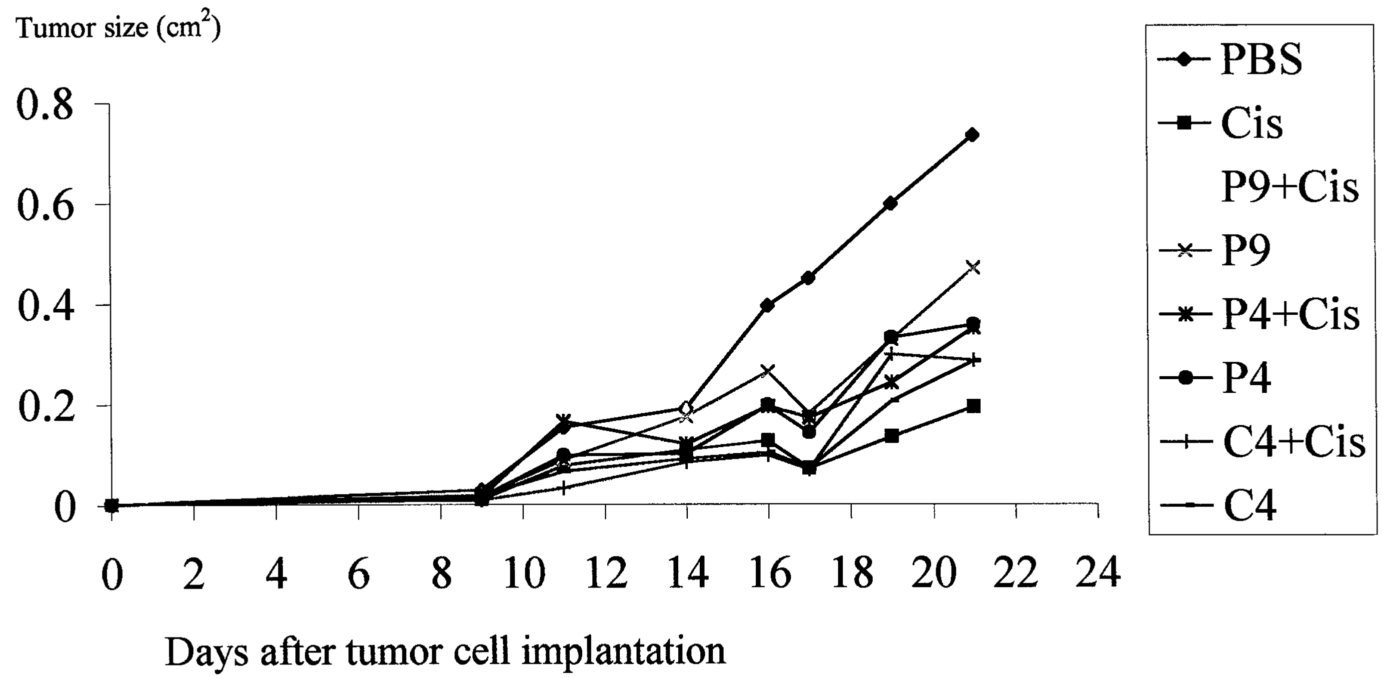
Fifis, T, Gamvrellis, A. Li, J, Proudfoot, O, Pouniotis, D., Brown, MP, Moghaddami, M, Osinski C, Bogdanoska V, McKenzie IFC, Plebanski M. Novel single dose vaccine induces strong cellular and humoral immunity and protection against tumour and malaria. *Nature Medicine* (submitted) 2001. (manuscript attached).

Xing, PX et al. Growth inhibitory antibodies for prostate cancer. (in preparation)

MengLiew EK, et al. Crosslinking dendritic cells by the mannose receptor leads to their activation. *J. Immunology* (in preparation).

CONCLUSIONS

The study is on track and will conclude this year. By the end of the year we should be able to demonstrate a) what is the best mode of delivery using mannan, DC Tag and possibly DNA; b) what are the best mixtures of antigens to use. These studies should be directly applicable to a clinical trial in humans.



LEGEND

Effect of inhibitory antibodies C4, P4 and P9 on the growth of a cancer cell line:

PBS: phosphate buffered saline; CIS: CIS-platinum; Tumor size: product of diameters (mm);

Days: after tumour cell modulation

Appendix II

Novel single dose vaccine induces strong cellular and humoral immunity and protection against tumour and malaria

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Key words: MHC class I, viral sized solid particles, IFNγ, antibodies, tumour regression, tumour protection, malaria protection, danger signal, generic vaccine carrier

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ABSTRACT

Immunisation with antigen conjugated to viral sized solid polysterene particles (VSSP) 0.04-0.05 μm diameter induced high numbers (>1/2000) MHC class I restricted CD8 T-cells. It also generated IgG antibodies, CD4 T-cells, regression of established tumours and protection against tumour challenge in mice. VSSP conjugated to *Plasmodium yoelli* antigens induced protection against challenge with lethal blood-stage malaria parasites. A single immunisation induced optimal immunity with strong responses maintained for 12 months. Virus sized compared to larger particles were targeted *in vivo* preferentially to dendritic cells expressing CD40 and CD86, which may explain their surprising effectiveness. VSSP offer a simple, potent and versatile single dose vaccine vehicle to induce strong, broad (humoral and cellular) and long-lasting immunity, and protection against widely different diseases. Specialised uptake of these viral sized particles by dendritic cells may provide insights into the evolution of non-self recognition.

INTRODUCTION

CD8 T-cells play a vital role in protective immunity against many intracellular pathogens and cancer but are notoriously difficult to induce at high levels for immunotherapy [1]. At present heterologous Prime-boost strategies and immunisation with *ex-vivo* antigen pulsed dendritic cells (DC) appear to be the best options [2-6]. However, the latter is expensive and logistically difficult, and multiple dose vaccines, such as Prime/boost may complicate administration to humans, particularly in the Third World. Safety concerns remain about the use of many live vaccine vectors. Here we show it is possible to efficiently deliver antigen to dendritic cells (DC) *in vivo* using viral sized solid particles (VSSP), leading to the subsequent induction of high numbers of antigen specific CD8 and CD4 T-cells and antibodies after a single immunisation. Immunity induced by VSSP protected completely against tumour and lethal blood-stage malaria challenge and cleared rapidly established tumours, providing a not only uniquely potent, but also highly versatile new vehicle for single dose vaccines.

In contrast to strategies targeting potential DC surface receptors [7], here a simple biological function of DC was explored, the uptake of antigen bound to solid particles within a narrow size range. Previous studies suggest optimal uptake and immunogencity requires carrier particles 1000 nm in diameter [2-6]. However, we found that polysterene particles 40-50 nm in diameter with antigen were up taken up more efficiently and were 500 fold better at inducing CD8 T-cells, CD4 T-cells and antibodies than larger or smaller particles, or protein made virus like particles (VLP) [4,8,9]. Both humoral and cellular responses were found up to 12 months after immunisation. Efficient *in vivo* uptake by DEC205+ CD40+ CD86+ lymph node cells for 40 nm but not 1000 nm particles was observed, and may provide a mechanism for the unusual potency of the virus sized particles, since this phenotype is associated with DC specialised in priming CD8 T cells [10,11]. This further suggests DC subsets may have evolved specifically to both recognise and stimulate immunity to viruses.

RESULTS

Immunogenicity of antigen conjugated to virus sized solid particles (VSSP)

Solid particles with absorbed antigen induce CD8 T-cell responses [12]. The suggested optimal particle size is 1μm. Virus like particles (VLP) made of protein 0.02-0.06μm diameter [4,8] also induce CD8 T-cells. We considered that antigen conjugated to viral sized polysterene particles could be highly immunogenic. Polystyrene beads of defined size (0.02, 0.04, 0.1, 0.5, 1 and 2μm) were conjugated to OVA and mice immunised intradermally (ID) twice (days 0 and 10). The frequency of T-cells secreting IFNγ to the MHC Class I H-2Kb restricted epitope SIINFEKL or to OVA, and serum OVA specific antibodies were assessed 10 days later (Fig. 1). A size specific distribution of immunogenicity was apparent, with optimal CD8 and CD4 T-cell (Fig. 1a) and IgG antibody (Fig. 1b and c) responses occurring at the 0.04 μm bead diameter size. No significant difference in immunogenicity was observed using beads 0.04 or 0.05 μm in diameter (not shown). Thus, beads within a viral size range induced optimal immunity. Similar responses were seen after a single ID immunisation, with 0.04 μm OVA conjugated beads inducing optimal immune responses (SIINFEKL specific T cell precursor frequency up to 1/1,500 spleen cells; mean 309 SFU +/- 111 SD by IFNγ ELISPOT, not shown). High IgG (Figure 1b) and CD8 T cell responses were present one year after a single immunisation and covalent conjugation to the beads was necessary for optimal immunogenicity (Figure 1c).

Protection against tumour High IFNγ producing and cytotoxic T-cell precursor frequencies are associated with protection against intracellular pathogens and tumours [1,2,4,6]. C57/B6 mice were immunised with 0.05μm beads-OVA intradermally and 30 days later injected with EG7 tumour cells expressing cytoplasmic OVA (Fig. 2). None (0/10) of the immunised mice developed tumours, whereas EG7 tumours were found in 10/10 of the non-immune controls. Similar results were observed after two immunisations (not shown). OVA alone or conjugated to 1 μm particles failed to confer similar levels of protection (Figure 2b). With view to a potential therapeutic use for such vaccines [13-15] solid EG7 tumours were established and then animals immunised with 0.05μm beads-OVA. Figure 2c shows that by 13 days after a single vaccination the mice in the experimental group cleared completely their tumours, regardless of the initial solid tumour burden, whereas in 5/6 non-treated mice tumours continued to progress. Thus the VSSP-antigen can protect from tumours and eradicate existing tumour masses after a single administration. Tumours re-appeared in some of the VSSP-OVA immunised animals in similar experiments followed until day 30 after challenge (4/8 mice). In contrast to tumours isolated at the same time from naïve mice, these new tumours lost the OVA SIINFEKL T cell epitope, as they were unable to stimulate a SIINFEKL specific T cell clone, or bind a H-2Kb-SIINFEKL complex specific monoclonal antibody in the absence of added SIINFEKL peptide (not shown). Thus strong immune selection pressure was induced by OVA-VSSP to eliminate SIINFEKL expressing tumour cells. These findings re-enforce the view that immunity to a single antigen expressed by tumour cells may not be sufficient to cure cancer in humans, and multiple antigens may have to be targeted by vaccines. New recombinant vehicles have to be constructed each time to test diverse antigen combinations when using VLP, viral or bacterial vaccine carriers. VSSP may provide a simpler vehicle for testing different antigen combinations since these can be directly bound onto the solid beads.

The development of VSSP for use against tumours was further supported by induction of protection against tumour lines expressing the breast cancer associated antigen, mucin 1 (MUC-1). In this model usually half of the DBA/2 mice develop tumours they are not able to control appropriately after subcutaneous challenge with P815 cells expressing MUC-1 (Tm211 cells). Immunisation with recombinant soluble fusion MUC-1 protein is not protective in this model[13-15]. By contrast 10/10 of the mice immunised once with VSSP-fusion MUC-1 protein controlled the tumours (Fig 2d). Immunisation with MUC-1 VSSP particles induced 1/5000, 1/11,111 and 1/5,555 IFNγ producing T cells to the weak MUC-1 CTL epitope STAPPAGVH, compared to <1/50,000 for each of 4 mice immunised with this protein alone or in Complete Freunds Adjuvant.

Protection against malaria Single dose vaccines may be particularly useful for large-scale use against infectious pathogens in the Third World. To further test the versatility of VSSP as a single dose vaccine carrier we tested protection in a radically different model, lethal blood-stage malaria challenge. *Plasmodium yoelii* infected red-blood cells were lysed, conjugated to VSSP and injected intra-dermally into mice. Protection was induced against subsequent lethal parasite challenge, where infected lysate alone, VSSP-OVA or VSSP conjugated to non-infected murine RBC lysate failed to induce protection (Fig 2e and f).

Viral size particles localise to a subset of dendritic cells in vivo The strong immunogenicity of VSSP conjugated antigen could reflect efficient uptake by professional APC. Fluorescent beads-OVA 0.02, 0.04, 0.1, 0.5, 1 and 2 µm in size were injected ID into the footpad of C57/B6 mice and draining popliteal lymph node collected 10 days later for FACScan analysis. Particles 0.04-0.1 µm in size were found 2-10 fold more abundantly in LN cells than any other sized particle (Fig. 3). 0.04-0.1 µm particles were taken up by macrophages (F4/80+) and by dendritic cells (DEC205+). However, uptake of 0.04-0.1 µm beads was twice as efficient for the latter (Fig. 2). To define further the phenotype of the cells that take up these virus sized particles, LN cells were dissected rapidly (48h hours) after ID immunisation with fluorescent 0.04 or 1 µm beads-OVA and stained for molecules expressed on subsets of professional APC. Most 0.04 µm-bead OVA positive cells expressed DEC205 and CD40 (>60%) and CD86 (>40%) (Fig. 2). By contrast, at this early time-point, 1µm beads-OVA were most abundant in F4/80 and CD80 expressing cells. These results suggest 0.04 µm beads_OVA may be more immunogenic than 1 µm not only because they localise 10X more efficiently to the lymph node, but because they were localised rapidly by a different antigen presenting cell subset. Consistent to this, in animals co-immunised with red 0.04 µm and green 1 µm OVA conjugated particles most 0.04 µm were found in a cell population which did not take up 1 µm beads, but not vice-versa (Figure 3c). Preliminary experiments also indicate a different mechanism of uptake by DC *in vitro* for 0.04 µm to 1 µm beads (not shown). Dendritic cells expressing CD40 and CD86 have been defined as potent stimulators of CD8 T-cell priming [10,11]. Rapid targeting of 0.04 µm beads to these cells may explain efficient VSSP induction of high CD8 T-cell levels.

Dendritic cells can use alternative MHC class I processing pathways for particulate antigen[4,8,16,17] Virus like protein particles (VLP) are processed by a TAP independent pathway [4,8,16,17]. TAP-/- C57/BL mice were immunised with OVA-0.04μm polystyrene particles. No T-cell responses above background were detected to SIINFEKL in TAP-/- animals (3/3 animals), despite significant responses after immunisation with DC pulsed exogenously with SIINFEKL (2/2 animals) (not shown). Therefore, processing for MHC class I presentation of VSSP was not TAP independent like VLP, suggesting a novel alternative pathway for processing of virus sized immunogens.

DISCUSSION

The present study shows that a single dose of antigen conjugated to solid particles 40-50 nm in size can induce CD8 T-cell responses comparable to the most potent methods currently available (>500 IFNγ SFU/million spleen cells) [2,3,5,6,18,19]. In previous studies, a direct comparison of CD8 T-cell inducing protocols including the adjuvants IFA, NACO, QS21 and AF, as well as lipopeptides, Ty VLP, DNA, Salmonella, Vaccinia and Copak as carriers, suggested lipopeptides and Ty VLP were the most immunogenic. Furthermore, only Ty VLP induced significant responses after a single dose corresponding to 50 +/- 30 SD IFNγ SFU/million spleen cells [5](Plebanski, unpublished). In the present study 10 times more CD8 T-cells were elicited with VSSP. Moreover, a single immunising dose of VSSP induced complete protection against tumour cell challenge and clearance of existing tumours. Substantial antibody responses were also elicited after VSSP immunisation. A single VSSP immunisation induced protection against lethal malaria parasite challenge. VSSP may provide a simple, versatile and powerful approach for human vaccine development. The targeting of whole recombinant antigens for MHC class I and II presentation is likely to induce T-cells to multiple epitopes, extending the potential use of such vaccines in different human populations. Multi-stage malaria vaccines for use in endemic areas may become a reality with the development of such versatile single dose vehicles.

The ability of VSSP within a narrow size range to induce singularly high CD8 T-cell levels could be the consequence of efficient uptake by APC or by a potent APC subset, targeting to the MHC class I processing pathway and/or direct stimulation of APC function. Uptake of 0.04-0.1 μm particles was found to be generally enhanced in the lymph node, compared to other sizes. Indeed it was 10 fold higher than the previously described immunogenic 1 μm particles. This enhanced uptake was tracked down to increased frequencies of particle positive DEC205+ CD40+ CD86+ cells. This DC phenotype is uniquely efficient in CD8 T-cell priming [2-6]. Recognition of size may thus have co-evolved in DC with ability to prime CD8 T cell responses. In this context, it is interesting that the optimal immunogenic size range identified in our study (40-50 nm) overlaps with that of many viruses, including a variety of Flaviviruses, Arboviruses, Orbiviruses and

Reoviruses (35-80nm). Antigens conjugated to VSSP elicited surprisingly broad (humoral and cellular) and strong responses (high T-cell frequencies) suggesting the immune system may be geared to react fully to particles of this viral size. Preferential uptake of particles of 40-100 nm in size by APC, and location in cells expressing potent co-stimulatory molecules (CD40 and CD86), may provide a mechanism whereby the immune system can react to dangerous stimuli via size clues. Such 'danger' clues can be incorporated into vaccines [20,21]. It probably is no coincidence that the effective inert vaccine formulations such as VLP and ISCOMS are of the same size range as VSSP. Viral sized solid particles were superior to VLP or ISCOMS in high and long lasting numbers of immune cells after a single dose [3,4,9,22,23]. Covalent conjugation of antigen and core particle stability may maintain VSSP integrity prior to processing inside the APC. By contrast, ISCOMS are mainly lipid with the propensity to fuse with cell membranes, not necessarily those of the APC. Self-assembling protein VLP are unstable when mixed with adjuvants, and may also have limited stability *in vivo*. Viral sized solid particles may be particularly useful since they offer a single dose intra-dermal immunisation protocol, potentially easy to administer to humans. There is no need for the construction of recombinant vehicles with VSSP, which may allow the comparative testing of large numbers of existing soluble antigens for their ability to induce both CD4 and CD8 T cell responses.

MATERIALS AND METHODS

Animals H-2Kb C57BL/6 and TAP-/- and H-2Kd DBA/2 6-8 week old mice were bred in house. 100 μg of antigen was immunised intradermaly (ID) in the hind footpads.

Bead-antigen conjugation Fluorescent (wavelegth 488 nm = green; 580 nm red) carboxylated polystrene fluospheres 0.02-2μ from Molecular Probes and non-fluorescent carboxylated polystrene microspheres from Polysciences adjusted to 2% solids were mixed 1:1 (vol:vol) with 2 mg/ml of Ovalbumin (OVA, Grade III, Sigma), recombinant MUC-1 fusion protein (ref) or *Plasmodium yoelii* infected red blood-cell lysate (generated as below) in 0.05M MES ([2-N-morpholino] ethane sulfonic acid) buffer pH 6.0 for 15 min. 4 mg/ml 1-Ethyl-3-(3-DimethylAminopropyl) carbodiamide added and the pH adjusted to 6.5 with NaOH and rocked for 3 hours. 100 mM glycine was added for 30 min before overnight dialysis against cold PBS against membranes of 300 kD pore size (Spectrum Laboratories Inc., Australia). <3 EU LPS was detected in OVA or beads used for injection. Efficiency of conjugation quantified using iodinated OVA ranged 24-95% (50-85% for the 0.04 μm size). Conjugated particles were stored at 4^0 C and sonicated for 15 min before use.

ELISPOT IFNγ assays 0.2-1x10^6 spleen cells were incubated in R10 alone or with SIINFEKL at 2.5 μ g/ ml or OVA at 25 μg/ ml for 18 hours on mixed acetate plates (MAHA Millipore) coated with anti-murine IFNγ mAb (R4), (EACC) as described [4]. Duplicate or triplicate wells were set up for each condition. Cells were discarded and plates incubated 2 hours with anti-murine IFNγ mAb-biotin (XMG.21-biotin,Pharmigen, CA, USA), followed extravidin-alkaline phosphatase (AP) at 0.1 μg/ml (Sigma). Spots of activity were detected using a colorimetric AP kit (Biorad, Hercules, CA, USA) and counted using a dissection microscope. Data are presented as mean spot forming units (sfu) per million cells +/- standard deviation (SD).

Phenotype analysis Monoclonal antibodies to CD40, CD80, CD86, CD8a and CD4 directly conjugated to phycoerythrin (PE) were from Pharmigen. Binding of mAbs from hybridoma supernatants to DEC205, F4/80, CD11c and CD11b was revealed with PE conjugated mouse anti-rat (Pharmigen). Cells dissected from lymph nodes were incubated in PBS 10% normal human serum 30 mins on ice with each antibody, washed and analysed by FACScan (Becton&Dickinson).

Uptake of OVA-fluorescent beads by FACScan was confirmed by confocal microscopy (not shown).

ELISA Polyvinyl chloride micro-titre plates coated with OVA (10 μg/ml in 0.2 M $NaHCO_3$ buffer, pH 9.6) were blocked with 2% bovine serum albumin (BSA) PBS 1 hour at 37°C, washed with PBS 0.2% Tween 5 times and incubated 2 hours at room temperature (RT) with mouse sera. Plates were washed 15 times and incubated with horseradish-peroxidase-conjugated sheep anti-mouse Ig, IgG, IgM or IgA (Selinus, AUST) for 1 hour at RT. Plates were washed again and developing buffer added (5 ml of ABTS buffer, 100 μl ABTS stock and 4 μl H_2O_2) for 30 mins. Plates were read on an EL312e microplate reader at 405nm.

Tumour protection and regression studies C57BL/6 mice were immunised with 100 μg OVA-0.05 μm particles or left untreated and 30 days later injected subcutaneously (SC) with $5x10^6$ OVA expressing EG7 cells. Tumours were measured using calipers at right angles. Animals with tumours >500 mm^2 were sacrificed. Similar experiments used MUC-1 fusion protein-0.05μm beads to immunise DBA/2 mice and SC challenge with $5x10^7$ Tm211 tumour cells expressing MUC-1[13-15]. For regression studies, C57BL/6 mice were given $5x10^6$ EG7 cells SC, and 8 days later divided into groups of similar tumour size distribution. One group was untreated and the other immunised with OVA-0.05 μm beads. Tumours measured as above. The p value for the Fisher exact test on chi square comparisons of tumour incidence is shown.

Malaria protection Blood was collected from C57BL/6 mice infected with *P. yoelii 17XL* at 50% parasitemia. Red blood cells (RBC) recovered after centrifugation 800g for 15 min were freeze/thawed three times and sonicated (lysate). Lysate was conjugated to 0.05 μm particles as above. Normal RBC lysate conjugated to particles was used as control. All mice were injected ID and challenged two weeks later intra-peritoneally with 1,000,000 *P. yoelii 17XL* infected RBC. The p value for the Fisher exact test on chi square survival comparisons is shown.

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FIGURE 1 Identification of the optimal immunogenic particle size

PANEL A Induction of IFNγ producing T-cells by immunization with OVA conjugated to beads of different sizes. C57/B6 mice were immunised intradermally (ID) twice (14 days interval) with OVA conjugated to 0.02,0.04,0.1,0.5,1 or 2 μm size beads and spleen T-cell responses to SIINFEKL or to OVA assessed 10 days after the booster immunisation by IFNγ ELISPOT. Reactivity to OVA was eliminated >75% by CD4+ cell depletion (not shown). One of three similar experiments is shown. Two mice per group were immunised for each bead size. The average values of spot forming units (SFU) per million cells +/- standard error (SE) for each mouse is shown. The frequency of SIINFEKL-specific cells with cytotoxic activity by limiting dilution analysis (LDA) correlated with IFNγ secreting T-cells (R^2=0.95, not shown). Conjugation efficiency: 0.02 μm, 95%; 0.04 μm, 69%; 0.1 μm, 52%; 0.5 μm, 44%, 1 μm, 30% and 2 μm, 24%. 1 μm–OVA beads induced similar T cell responses at either 50, 100 or 1000 μg/mouse, and the same was observed for 0.04 and 0.1μ-OVA beads (not shown). **PANEL B Antibody production induced by immunisation with OVA conjugated to beads of different sizes.** Serum was collected 10 days after the booster immunisation and dilutions tested for OVA specific Ig by ELISA. The mean optical density at 405nm +/- SD for each group receiving 0.02, 0.04, 0.1, 0.5, 1 or 2 μm size OVA-bead immunisation is plotted. Naïve serum is shown as a negative control. One of two similar experiments is shown. **PANEL C Induction of long lasting antibody responses by a single immunisation.** C57/B6 mice were immunised once with OVA conjugated to 0.05μm beads and sera collected at different time-points. The mean optical density at 405nm +/- SE for each group of four animals in OVA specific IgG ELISA is shown. Naïve sera is shown as negative control. One of two similar experiments is shown. Similar ELISA results were obtained for total Ig and no IgM or IgA was detected (not shown). OVA alone failed to induce IgG responses over PBS immunised animals and OVA in Complete Freunds Adjuvant (CFA) induced IgG responses a log higher than single dose 0.05 μm beads-OVA (not shown). **PANEL D Induction of long lasting high levels of IFN□producing T-cells by a single immunisation with 0.5 μm beads OVA** C57/B6 mice were immunised ID once with OVA conjugated to 0.05 μm beads (black or chequered bar), soluble OVA in PBS (white bar) or with OVA mixed in with 0.05 μm beads (grey bar). Precursor frequency of SIINFEKL reactive spleen T-cells was assessed 10 days later (back, white and grey bars) or 12 months later (chequered bar) by IFNγ ELISPOT. Four mice were tested per group and one of two similar experiments is shown. Average values of spot forming units (SFU) per million cells +/- standard deviation (SE) are shown for each group. In similar experiments using 10 times less antigen (10 μg VSSP-OVA) a single immunisation induced 102 +/- 56 SIINFEKL specific spleen cells per million (n=4). Cytotoxic T-cell responses in standard Chromium release assays were also observed 10 days after a single immunisation (>50% Specific lysis for 3/3 animals at E:T ratio 20:1; not shown)

FIGURE 2 Protection against cancer and malaria

PANEL A Complete protection against challenge with the EG7 cell line by a single immunisation with OVA conjugated to 0.05 µm particles C57/B6 mice were immunised ID with OVA conjugated to 0.05 µm particles or left untreated. 30 days later mice were challenged with EG7 cells subcutaneusly (SC) and tumour growth measured with electronic digital calipers. Data is presented as the individual tumour growth curves for 10 animals in each group. One of two similar experiments is shown. The difference in the frequency of tumours at all time-points was highly significant ($p<0.00001$) **PANEL B Soluble OVA and 1 µm-OVA beads fail to induce comparable protection to 0.05 µm-OVA beads.** C57/B6 mice were immunised ID with OVA conjugated to 0.05 µm or 1 µm beads, soluble OVA or left untreated and then challenged as above. Data is presented as the individual tumour sizes at day 10 for 8 animals in each group. One of two similar experiments is shown. The difference in the frequency of tumours between the 0.05 µm-OVA bead group and each one of the other groups was significant: $P=0.0001$ vs. naïve; $p=0.0007$ vs. soluble and $p=0.0035$ vs. 1 µm bead-OVA. **PANEL C Regression of established EG7 tumours** Tumours were induced in C57/B6 mice by SC injection with EG7 cells. Animals were distributed into two groups of similar tumour sizes on day 8 and one group left untreated (Naïve) and the other immunised ID once in the footpad with OVA conjugated to 0.05 µm particles (Immunised). Data is presented as the individual tumour growth curves for 6 animals in each group after immunisation. One animal in each group was culled at day 9 due to tumour size above 500 mm (Naïve group) or suspected disseminated tumour (Immunised). The experiment was terminated culling all the animals on day 16. Only one animal was still alive in the naive group, and it had a tumour of 16 mm. All animals in the immunised group were healthy and without tumours. The difference in the frequency of regressed tumours between the two groups was significant ($p=0.04$). One of two similar experiments is shown. **PANEL D Induction of protective immunity to the breast cancer antigen MUC-1.** DBA/2 mice were immunised ID with MUC-1 fusion protein conjugated to 0.05 µm particles. 30 days later mice were challenged with Tm211 cells SC and tumour growth measured as above. Data is presented as individual tumour growth curves for 10 animals per group. The difference in the mice that did and did not control tumour growth by day 26 was significant ($p=0.043$) **PANEL E Survival of mice to lethal malaria challenge after VSSP immunisation.** Parasite lysate was generated by freeze-thaw and sonication of *P. yoelii 17XL* infected red-cells and C57/B6 mice immunised ID with lysate (empty squares), lysate conjugated to 0.05 µm beads (full squares), 0.05µ beads-OVA (full circles) or left untreated (empty circles). **PANEL F** Beads conjugated to non-parasitised murine RBC (empty diamonds) were also used as controls in some of the experiments. Two weeks later mice were challenged with live *Plasmodium yoelii 17XL*. 5 or 10 animals were challenged per group (Panels E and F respectively) and survival for representative experiments is shown. Significant differences were observed in the analysis of the cumulative survival comparing mice immunised with parasitised RBC lysate conjugated to beads and untreated (P=0.005); parasitised lysate alone (p=0.04) or bead conjugated non-parasitised lysate immunised mice (p<0.00001). A total of 85 mice were used in these experiments, 30 for parasitised RBC lysate, 10 for naïve, 5 for beads-OVA, 10 for parasitised RBC lysate and 30 for non-parasitised RBC lysate conjugated to beads.

FIGURE 3 ***Uptake by antigen presenting cells***

PANEL A 0.04-0.1 μm particles are preferentially found in draining LN cells after ID immunisation C57/B6 mice were immunised intradermally (ID) in the footpad twice (14 days interval) with OVA conjugated to 0.02,0.04,0.1,0.5,1 or 2 μm size flourescent (FITC) beads and the draining popliteal lymph nodes (LN) dissected 10 days after the last immunisation. The percentage of FITC+ LN cells was assessed by FACScan for triplicate samples of LN cells for each group. The data is presented as the mean FITC % uptake for +/- the standard error (SE). One of 3 similar experiments is shown. Lymph node cells taking up the FITC particles as above were assessed for expression of the dendritic cell (DC) marker DEC205 or the macrophage associated marker F4/80 by staining with specific mAbs. Data is presented as the % expressing DEC205 or F4/80 of the total fluorescent particle positive cells. DEC205+ and F4/80+ cells were 51% +/- 12 SD and 7% +/- 3 SD of popliteal LN cells, respectively. Fluobead+ cells were not present in cervical, axillar or mesenteric LN or the thymus but 0.48% of inguinal LN were 0.04 μm fluobead-OVA positive (not shown). The same amount of solid mass and total protein was injected for each bead size. The number of particles in that equivalent mass for 0.02,0.04,0.1,0.5,1 or 2 μm size flourescent beads was 10^{13}, 10^{13}, 10^{12}, 10^{11}, 10^{10} and 10^{9}, respectively. Similar relative results were obtained when comparing 0.04 and 1 μm beads at 10^{9} each (not shown). **PANEL B Phenotypic characterisation of APC taking up 0.04 compared to 1 μm particles *in vivo*** C57/B6 mice were injected in the footpad with 50 μl of 0.04 or 1 μm fluobeads-OVA. Draining popliteal LN were analysed 48 hours later for co-staining of bead positive cells with cell markers of activation and antigen presenting cell lineage, the mean +/- SE for 3-14 mice/marker is shown. 0.04 and 1 μm fluobead+ cells had significantly different expression of DEC205, F4/80, CD40, CD80 and CD86 (p<0.05). **PANEL C *In vivo* co-localisation of 0.04 and 1 μm beads** OVA C57/B6 mice were injected with ID with 25 μl green-fluorescent 1 μm (FL-1) or red-fluorescent 0.04 μm (FL-2) beads alone (bottom and top left panel, respectively) or mixed together (25μl+25μl) (right top panel). Draining lymph nodes were dissected 48 hours later and cells analysed by FACscan. One of two similar experiments is shown.

FIGURE 1

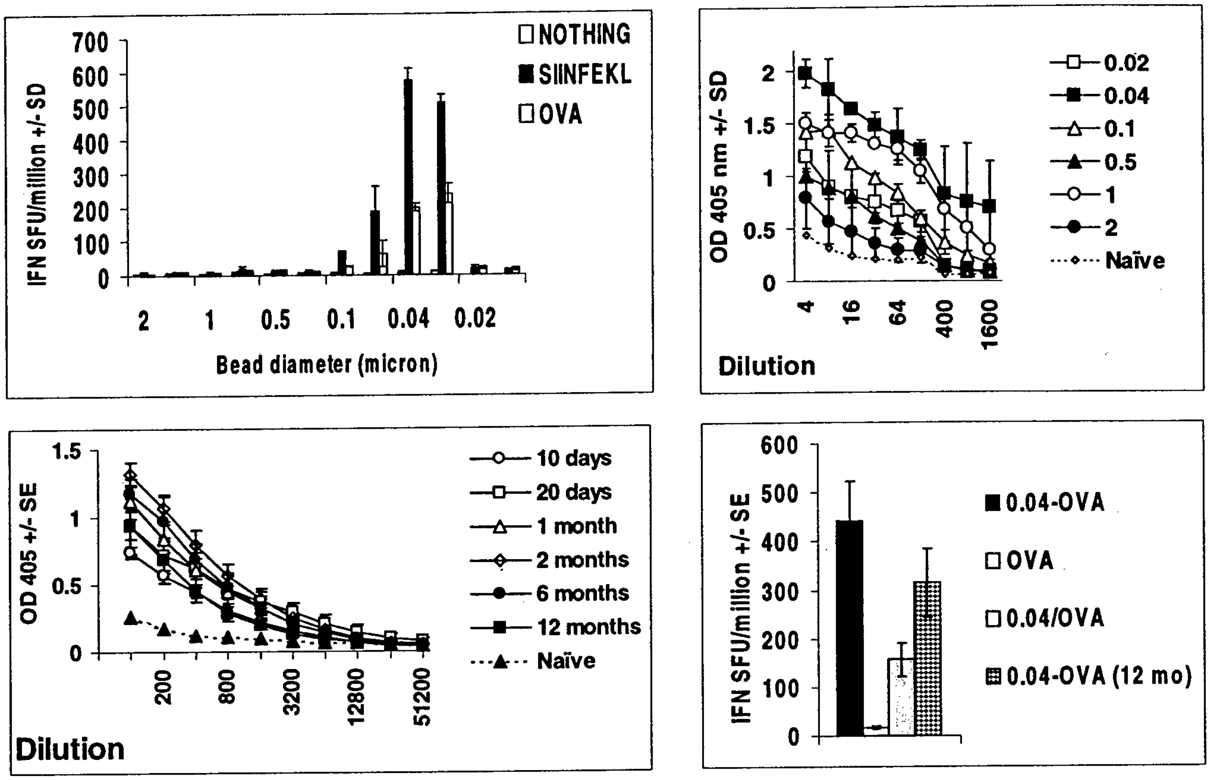

IFN SFU/million +/- SD
700
600
500
400
300
200
100
0
NOTHING
SIINFEKL
OVA
2
1
0.5
0.1
0.04
0.02
Bead diameter (micron)
OD 405 nm +/- SD
2
1.5
1
0.5
0
4
16
64
400
1600
Dilution
0.02
0.04
0.1
0.5
1
2
Naïve
OD 405 +/- SE
1.5
1
0.5
0
200
800
3200
12800
51200
Dilution
10 days
20 days
1 month
2 months
6 months
12 months
Naïve
IFN SFU/million +/- SE
600
500
400
300
200
100
0
0.04-OVA
OVA
0.04/OVA
0.04-OVA (12 mo)

FIGURE 2

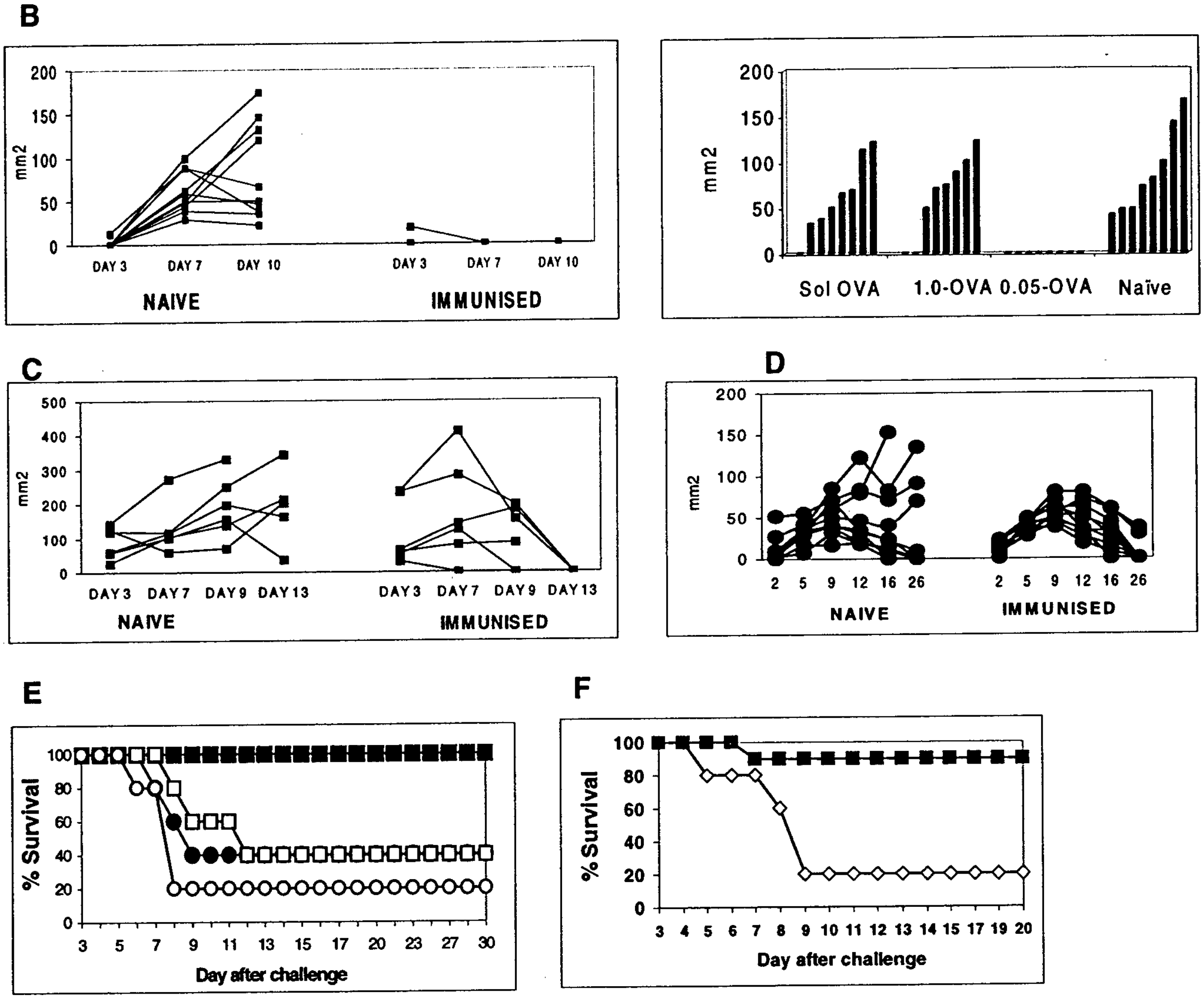
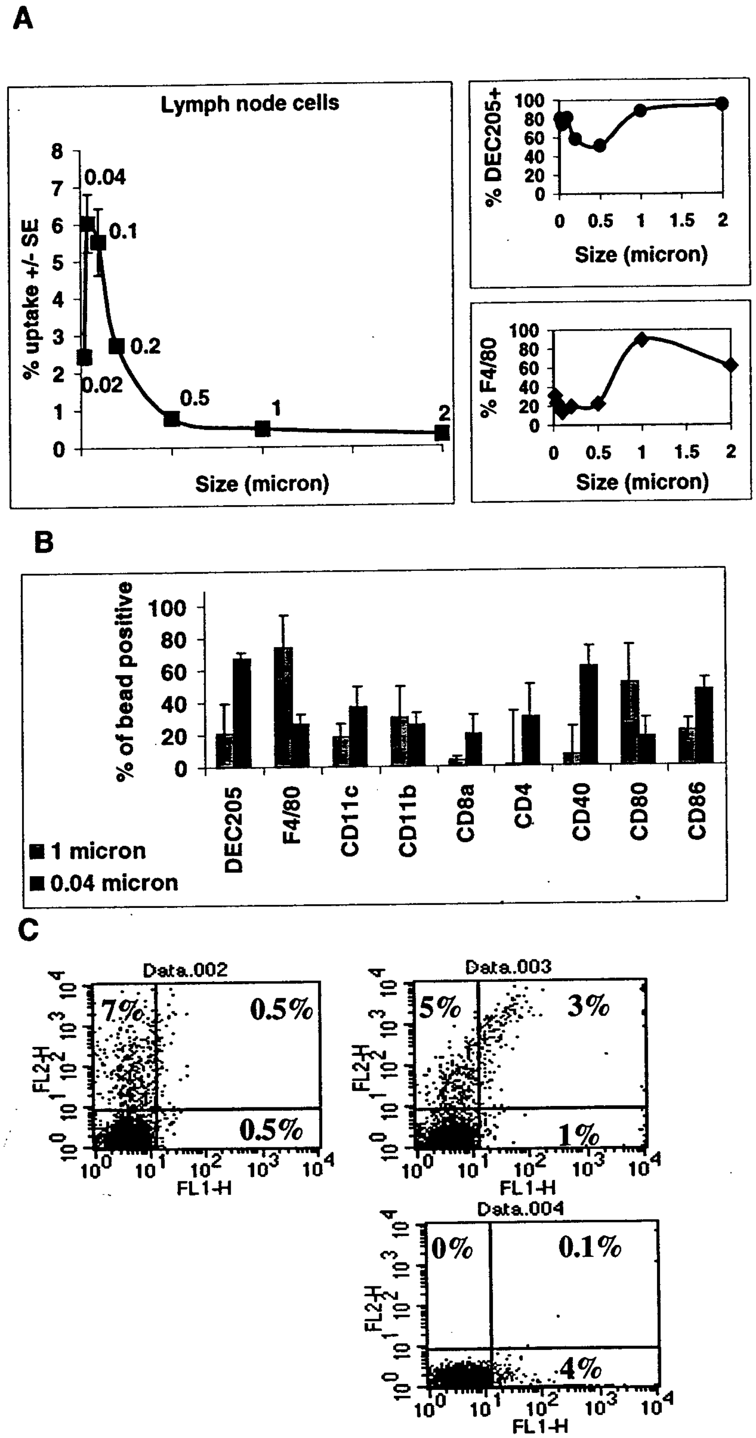

B
mm2
DAY 3
DAY 7
DAY 10
NAIVE
IMMUNISED
Sol OVA
1.0-OVA
0.05-OVA
Naive
C
DAY 3
DAY 7
DAY 9
DAY 13
NAIVE
IMMUNISED
D
2 5 9 12 16 26
NAIVE
IMMUNISED
E
% Survival
Day after challenge
F
% Survival
Day after challenge

FIGURE 3


A
Lymph node cells
% uptake +/- SE
0.04
0.1
0.2
0.02
0.5
1
2
Size (micron)
% DEC205+
Size (micron)
% F4/80
Size (micron)
B
% of bead positive
DEC205
F4/80
CD11c
CD11b
CD8a
CD4
CD40
CD80
CD86
1 micron
0.04 micron
C
Data.002
7%
0.5%
0.5%
FL2-H
FL1-H
Data.003
5%
3%
1%
FL2-H
FL1-H
Data.004
0%
0.1%
4%
FL2-H
FL1-H