The Journal of Immunology

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J Immunol 2004; 173:3148-3154; ;
doi: 10.4049/jimmunol.173.5.3148
http://www.jimmunol.org/content/173/5/3148

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Size-Dependent Immunogenicity: Therapeutic and Protective Properties of Nano-Vaccines against Tumors[1]

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Infection can protect against subsequent disease by induction of both humoral and cellular immunity, but inert protein-based vaccines are not as effective. In this study, we present a new vaccine design, with Ag covalently conjugated to solid core nano-beads of narrowly defined size (0.04–0.05 μm) that localize to dendritic cells (DEC205$^+$ CD40$^+$, CD86$^+$) in draining lymph nodes, inducing high levels of IFN-γ production (CD8 T cells: precursor frequencies 1/5000 to 1/1000) and high Ab titers in mice. Conjugation of Ag to these nano-beads induced responses that were significantly higher (2- to 10-fold) than those elicited by other bead sizes, and higher than a range of currently used adjuvants (alum, QuilA, monophosphoryl lipid A). Responses were comparable to CFA/IFA immunization for Abs and ex vivo peptide-pulsed dendritic cell immunization for CD8 T cells. A single dose of Ag-conjugated beads protected mice from tumors in two different model challenges and caused rapid clearance of established tumors in mice. Thus, a range of Ags conjugated to nano-beads was effective as immunogens in both therapeutic and prophylactic scenarios. ***The Journal of Immunology*****, 2004, 173: 3148–3154.**

CD8 T cells play a vital role in protective immunity against many intracellular pathogens and cancer, but are notoriously difficult to activate with vaccination and immunotherapy (1). Vaccines that require a specific immunization sequence with repeated doses, such as Prime-boost are very efficient (1), but complicated to administer, particularly in the Third World, where logistic considerations of vaccine distribution are critical (2). A simple inert carrier (nonlive) able to generate strong protection after a single dose could be useful for a variety of applications.

Solid inert beads with surface-adsorbed Ag have previously been used to stimulate CD8 T cell responses, with an optimal bead diameter size of 1 μm, and <0.5 μm reported as inferior in targeting Ag for MHC class I-restricted presentation to T cells (3). However, other particulate immunogenic carriers such as immune-stimulating complexes (ISCOMS)[3] and virus-like particles (VLP) as well as many common viruses have a size range of 0.03–0.2 μm in diameter. Although in most vaccine protocols adjuvants are essential to provide appropriate danger signals for successful induction of immune responses (4), particulate vaccines appear to induce immune responses without adjuvants (5, 6). Usually, and in contrast to natural infections, vaccines with adjuvants able to induce high CD8 T cell responses do not generate high Ab levels and vice versa (1, 7). In this study, we systematically explored inert carrier size within both a viral (<0.5 μm) and a bacterial range (>0.5 μm), and examined Ag uptake efficiency and in vivo localization of beads in the lymph node (LN) after injection, as potential parameters of immunogenicity. Surprisingly, the optimal carrier bead size was narrowly defined, ~0.04–0.05 μm, in the viral range. Moreover, Ag conjugated to this bead size was able to elicit combined Ab and CD8 T cell immunity comparable to leading adjuvants for each arm of the immune response. The unusual potency of this novel nano-vaccine approach was demonstrated by the ability to clear large established tumor masses within 2 wk after a single injection.

Materials and Methods

Animals

H-2K^b C57BL/6 6- to 8-wk-old mice were bred at the Austin Research Institute (Victoria, Australia).

Bead-Ag conjugation

Carboxylated polystyrene microspheres (Polysciences, Eppelheim, Germany) with or without fluorescent labels (Molecular Probes, Eugene, OR), ranging in size from 0.02 to 2 μm, were adjusted to 2% solids, mixed 1:1 (v:v) with 2 mg/ml OVA (grade III; Sigma-Aldrich, St. Louis, MO), in 0.05 M MES buffer, pH 6.0, for 15 min. For covalent conjugation, 1-ethyl(3-dimethylaminopropyl) carbodiimide (Sigma-Aldrich) was added at 4 mg/ml, pH adjusted to 6.5 with NaOH, and preparations (mixed or conjugated) rocked for 3 h at room temperature. Glycine was added to 100 mM for 30 min before overnight dialysis in cold PBS using a 12-kDa membrane cutoff. After dialysis, volume was adjusted to maintain 1% solids and 1 mg/ml OVA. Endotoxin levels (<3 EU/ml in all final nano-bead preparations tested) were assayed by EML Consulting Services (Surrey Hills, Victoria, Australia). Efficiency of conjugation was determined in representative experiments by inclusion of trace amounts of ^{125}I-labeled OVA. After ^{125}I-labeled conjugation, 100-μl samples were taken and free OVA was removed by repeated centrifugation. Solids and PBS washings were counted to determine percentage of Ag conjugated. Conjugated beads were stored at 4°C and sonicated for 15 min before use. The same procedure was used to conjugate other Ags and peptides, including the human papilloma virus (HPV) strain 16 E7 protein-derived peptide, henceforth called E7.1 (RAHYNIVTF).

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Received for publication March 22, 2004. Accepted for publication June 25, 2004.

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[1] M.P. is a Howard Hughes International Scholar and Senior National Health and Medical Research Council Fellow. The work was funded by National Health and Medical Research Council, Australia, 181614 and 222302; Cancer-Council of Victoria v13; U.S. Army Breast Cancer Award DAMD17-99-1-9067; and PRIMA BioMed (Australia).

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[3] Abbreviations used in this paper: ISCOMS, immune-stimulating complexes; DC, dendritic cell; HPV, human papilloma virus; LN, lymph node; MPL, monophosphoryl lipid A; SFU, spot-forming unit; VLP, virus-like particle.

0022-1767/04/$02.00

Adjuvants and immunizations

A total of 2 mg/ml OVA in PBS was mixed for injection at 1:1 (v:v), with the adjuvants following in each case previously tested protocols (8) or the manufacturer's recommendations for optimal immunogenicity. Adjuvants used were: CFA (Sigma-Aldrich), QuilA (Superfos Biosector, Vedbaek, Denmark), 20 μg; monophosphoryl lipid A (MPL) + trehalose dicory nomycolate + cell wall skeleton adjuvant system (Sigma-Aldrich), abbreviated in text as MPL; alum, $Al(OH)_3$ (3% aluminum potassium phosphate, 0.17 M NaOH). Animals were boosted after 14 days with the same formulation, except IFA was used in place of CFA. Unless otherwise stated, 100 μg of Ag was administered intradermally. As a live adjuvant system, bone marrow-derived dendritic cells (DC) were prepared, as described (9), and pulsed with either 100 μg/ml OVA by overnight culture or 25 μg/ml SIINFEKL peptide for 3 h at 37°C and immunized at 1×10^6 cells per animal. Preliminary experiments testing different peptide-pulsing concentrations, times to pulse, and numbers of DC injected were conducted to induce optimal immunity (data not shown).

ELISPOT assays

As described previously (6), spleen cells were incubated with SIINFEKL at 2.5 μg/ml or OVA at 25 μg/ml for 18 h on plates (multiscreen mixed esther; Millipore, Billerica, MA) precoated with anti-IFN-γ mAb (clone R4; American Type Culture Collection, Manassas, VA). Cells were discarded, and plates were incubated with anti-IFN-γ mAb biotin (XMG.21 biotin; BD Pharmingen, San Diego, CA), followed by extravidin-alkaline phosphatase (Sigma-Aldrich), and spots were detected with an alkaline phosphatase kit (Bio-Rad, Hercules, CA). Data are presented as mean spot-forming units (SFU) per million cells ± SE.

Phenotypic analysis

Draining popliteal LN cells were isolated from mice treated by intradermal footpad injection with nano-beads (Ag coupled, with or without red or green fluorescent tags). These cells, as a single-cell suspension, were incubated on ice for 30 min with PE-conjugated mAb to CD40, CD80, CD86 (BD Pharmingen), or mAb hybridoma supernatants to DEC205, F4/80, followed by PE mouse anti-rat (BD Pharmingen) in cold PBS/10% FCS, washed three times with the latter, and analyzed by FACScan (BD Biosciences, San Jose, CA).

ELISA for anti-OVA Abs

Plates (96-well) coated with OVA (10 μg/ml in 0.2 M $NaHCO_3$ buffer, pH 9.6) were incubated with test or control mouse sera, followed by incubation with HRP-conjugated sheep anti-mouse-Ig (SILENUS Labs, Melbourne, Australia), and detected with ABTS. Titer was defined as the serum dilution at which the 405 nm OD was above the mean + 3 SDs of naive sera values.

Tumor protection and regression studies

Immunized and naive C57BL/6 mice were injected s.c. with 5×10^6 OVA-expressing EG7 cells (10). Tumors were measured using calipers at right angles. For immunotherapy studies, mice were divided into groups of similar tumor size distribution at 8 days posttumor injection. One group was left untreated and the other immunized with OVA/0.04-μm nano-beads. Tumors were measured as above for an additional 13 days. Note that different batches of EG7 cells were used in the protection and therapy experiments. A further tumor model was tested. E7.1 (sequence) peptide, alone or conjugated to 0.04-μm beads, was used to immunize C57BL/6 mice, and these were challenged s.c. 30 days later with 5×10^6 EL4 tumor cells expressing the E7 HPV strain 16 protein (11) (kindly provided by R. Tindle, University of Queensland, Brisbane, Australia).

Statistics

Values of p for differences between mean SFU by ELISPOT or mean inverse titer by ELISA for diversely immunized animal groups were determined using two-tailed Student's t test. Values of p for Fisher exact test on χ^2 comparisons of tumor incidence were calculated, where appropriate.

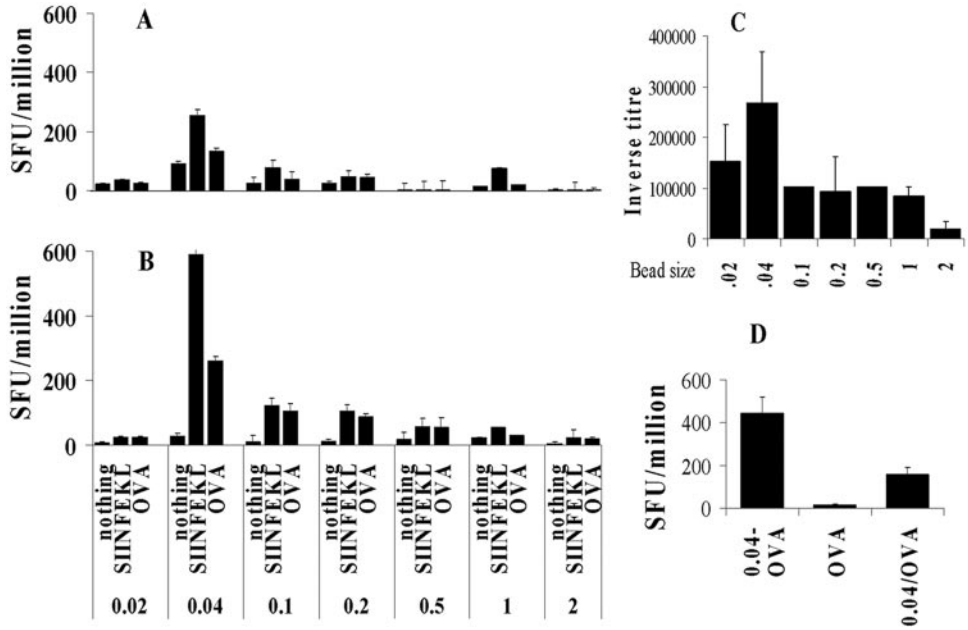

FIGURE 1. Bead size and immunogenicity: T cell responses. C57BL/6 mice were immunized intradermally once (*A*) or twice (*B*), 2 wk apart, with OVA conjugated to 0.02-, 0.04-, 0.1-, 0.5-, 1-, or 2-μm-size beads with 100 μg of OVA at 1% carrier solids for each bead size per animal. Spleen T cell responses to SIINFEKL or OVA were assessed 10 days after the last immunization by IFN-γ ELISPOT. One immunization: 0.02 ($n = 3$ animals), 0.04 ($n = 33$), 0.1 ($n = 13$), 0.2 ($n = 3$), 0.5 ($n = 3$), 1 ($n = 7$), or 2 μm ($n = 3$). After one immunization, although both 0.01- and 1-μm beads showed responses significantly greater than naive controls ($p < 0.02$), these responses were much lower than for 0.04-μm beads ($p < 0.0001$ compared with naive controls). For two immunizations (*B*), 0.02 ($n = 10$ animals), 0.04 ($n = 4$), 0.1 ($n = 10$), 0.2 ($n = 4$), 0.5 ($n = 4$), 1.0 ($n = 10$), or 2.0 μm ($n = 4$), the 0.04-μm beads gave higher responses ($p < 0.03$ when SIINFEKL responses with 0.04-μm beads were compared with other bead sizes or with naive controls). Ab responses (*C*): mice were immunized twice as above, serum collected 10 days after the second immunization, and dilutions tested for OVA-specific Ig by ELISA. The mean inverse titer ± SE for 3–13 animals per group is shown. Naive serum was used as negative control. In this figure, the 0.04-μm bead responses were only significantly different when compared with 0.1-, 0.5-, and 2-μm beads ($p < 0.05$). *D*, Mice were immunized with soluble OVA ($n = 2$), OVA mixed with beads (0.04/OVA, $n = 2$) or conjugated to beads (0.04-OVA, $n = 4$), as above, and spleen T cell responses to SIINFEKL were assessed 10 days later by IFN-γ ELISPOT. Precursor frequency is shown as mean SFU/million cells ± SE for each mouse. Responses for OVA-conjugated beads were significantly different to both soluble OVA and OVA mixed with beads ($p < 0.03$).

Results

Immunogenicity is dependent on bead size and covalent conjugation of Ag

OVA was conjugated to the surface of beads over a range of viral and bacterial sizes (0.02, 0.04, 0.1, 0.5, 1, and 2 μm), and mice were immunized once with the same volume of 1% bead solids for all sizes. T cell responses measured 10 days after one immunization with nano-beads/OVA were induced optimally using the 0.04-μm size, with many T cells responsive to OVA and to its MHC class I epitope SIINFEKL, as measured by IFN-γ production (Fig. 1*A*). The majority of animals (70%) had SIINFEKL-specific spleen cells at precursor frequencies between 1/5000 and 1/2000, as measured by IFN-γ ELISPOT, with 24% below and 6% above this range ($p < 0.05$ for SIINFEKL responses in the 0.04-μm bead group compared with other bead sizes and naive controls). None of the other sizes induced precursor frequencies above 1/5000. Two immunizations induced the same pattern of response (Fig. 1*B*) and also showed higher Ab titers with 0.04-μm beads 10 days after the second immunization (Fig. 1*C*). Similar T cell responses were obtained with 0.043- or 0.048-μm beads (hence called generically 0.04-μm nano-beads). Both high numbers of MHC class I-restricted T cells and Ab titers were therefore induced by immunization with Ag covalently conjugated to a narrowly defined size range of nano-beads, in the absence of adjuvants. The covalently conjugated Ag-bead preparation also promoted higher T cell responses than Ag simply mixed with the nano-beads or soluble Ag without the bead carrier ($p < 0.03$ for both comparisons) (Fig. 1*D*).

Quantitative considerations

Covalent conjugation attaches a higher concentration of Ag onto 0.04-μm beads (8×10^{-11} $\mu g/\mu m^2$) than simple adsorption (1×10^{-11} $\mu g/\mu m^2$) at the same bead to Ag ratio. Assuming that only the bead-conjugated Ag contributed to the induction of immune responses, it was conceivable that the high immunogenicity of 0.04-μm-size beads was due to particularly high total or per unit surface area amounts of Ag compared with beads of other sizes. However, at standard conjugation conditions (1% bead solids and 1 mg/ml Ag), the 0.02-μm beads conjugated more total Ag, and the 0.1- to 2-μm beads more Ag per unit surface area than the 0.04-μm beads (Fig. 2*A*), but only the 0.04-μm-size beads induced high cellular or humoral responses (Fig. 1). This suggested that an adequate dose of Ag conjugated to the 0.04-μm beads is necessary, but not sufficient for enhanced immunogenicity. To test the effect of varying the total amount of conjugated Ag, 0.04- and 1-μm beads were conjugated at the normal concentration (1% solids, 1 mg/ml Ag) and then diluted before immunization to give Ag doses of 0.35–70 μg/mouse (Fig. 2*B*). The 0.04-μm beads gave significantly stronger responses when equal doses of conjugated Ag were delivered, further indicating that the dose of Ag conjugated is not the sole determinant of the superior immunogenicity profile of 0.04-μm nano-beads. Other potential factors were therefore examined.

In vivo localization of beads

We followed the fate of different sizes of fluorescent OVA-conjugated beads after intradermal injection. At various intervals from 48 h to 14 days postimmunization, cells that had taken up beads were found in the draining LN. At both 48 h and 14 days, the 0.04-μm beads were found in more cells than other smaller or larger sized beads (Fig. 3*A*). Phenotypic analysis at 48 h of the LN cells showed preferential localization of the 0.04-μm beads to cells expressed the markers DEC205$^+$, CD40$^+$, and CD86$^+$. These markers are characteristic of the mature/activated DC subset of s.c. origin (Fig. 3*B*). In contrast, at 48 h, 1-μm beads localized preferentially to F4/80$^+$, CD80$^+$ cells representing a different, macrophage-like subset of APC (12), and 0.02-μm beads were found in CD40$^+$ cells, but not DEC205$^+$ or F4/80$^+$ cells (Fig. 3*B*). After 14 days, although fewer bead-positive cells were detected, the same phenotypic profile was seen in these cells, with the 0.04-μm beads continuing to localize preferentially to DEC205$^+$ and the 1-μm beads to F4/80$^+$ cells, with costimulatory marker expression similar to that seen at 48 h (data not shown). The results indicate that 0.04-μm nano-beads are efficient Ag carriers to the DC in the draining LN and that beads persist within LN cells for >14 days (considering both total LN uptake and cell phenotype).

The finding that the 0.04-μm beads preferentially localize to DCs in the draining LN suggests that beads of this size cause activation of DCs and migration from dermal sites. To investigate this, red 0.04-μm beads were mixed with green 1-μm beads and injected intradermally to determine whether this would enhance the localization of the larger particles to draining LN. Fig. 3*C* shows that there was enhanced uptake of larger beads into LN cells when mixtures were injected, compared with 1-μm beads injected alone (Fig. 3*C*, $p = 0.0009$). OVA-conjugated 1-μm beads mixed with

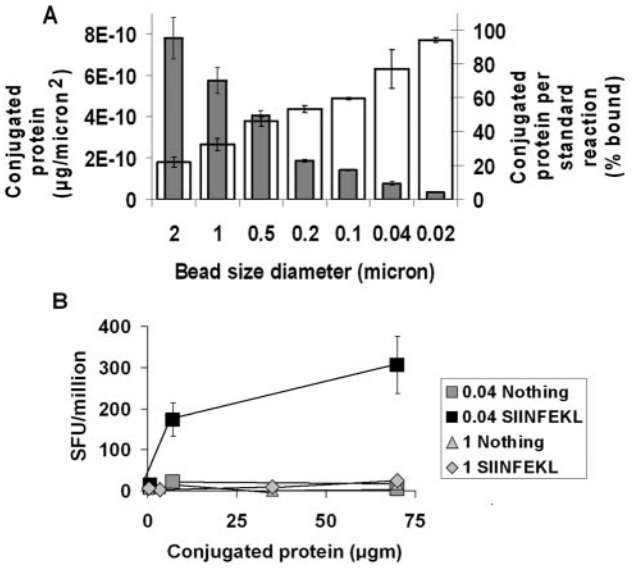

FIGURE 2. Effect of bead size on Ag conjugation. *A*, The amount of conjugated Ag/surface area (▨) and percentage of Ag conjugated per standard reaction (□) are shown for each bead size. Compared with 0.04-μm beads, 0.2-, 0.5-, 1-, and 2-μm beads conjugated more Ag/surface area, but 2- and 1-μm beads took up less percentage of Ag/standard reaction ($p < 0.05$). The 0.04- and 0.02-μm beads took up more percentage of Ag/reaction than beads ≥0.5 μm, with 0.02 μm slightly more reactive than 0.04-μm beads ($p = 0.052$). Ag conjugation was determined by the inclusion of trace ^{125}I-labeled OVA, as in *Materials and Methods*. The mean ± SE for the number of conjugation experiments for each bead size is shown: 0.02 ($n = 2$), 0.04 ($n = 6$), 0.1 ($n = 2$), 0.2 ($n = 2$), 0.5 ($n = 2$), 1 ($n = 3$), or 2 μm ($n = 2$). *B*, Beads were diluted after conjugation, and mice were immunized with decreasing amounts of OVA conjugated to 0.04-μm compared with 0.1- and 1-μm beads. Conjugation was conducted as normal with 1% solids: 1 mg of Ag/ml. Ag dose was adjusted by dilution of the conjugates before immunizing the animals. The mean IFN-γ SFU/million cells ± SE per group of animals for each condition is shown: 0.04 μm, 70 μg ($n = 7$ animals), 7.0 μg ($n = 5$), and 0.7 μg ($n = 4$); 0.1 μm, 60 μg ($n = 2$), 6 μg ($n = 6$), and 0.6 μg ($n = 6$); 1 μm, 60 μg ($n = 2$), 35 μg ($n = 5$), 3.5 μg ($n = 2$), and 0.35 μg ($n = 2$). The 0.04-μm beads gave significantly better results, $p < 0.001$ compared with naive controls for 70- and 7-μg doses of 0.04-μm beads/OVA, $p > 0.05$ for other doses, and for all doses of 1- or 0.1-μm OVA beads compared with naive controls.

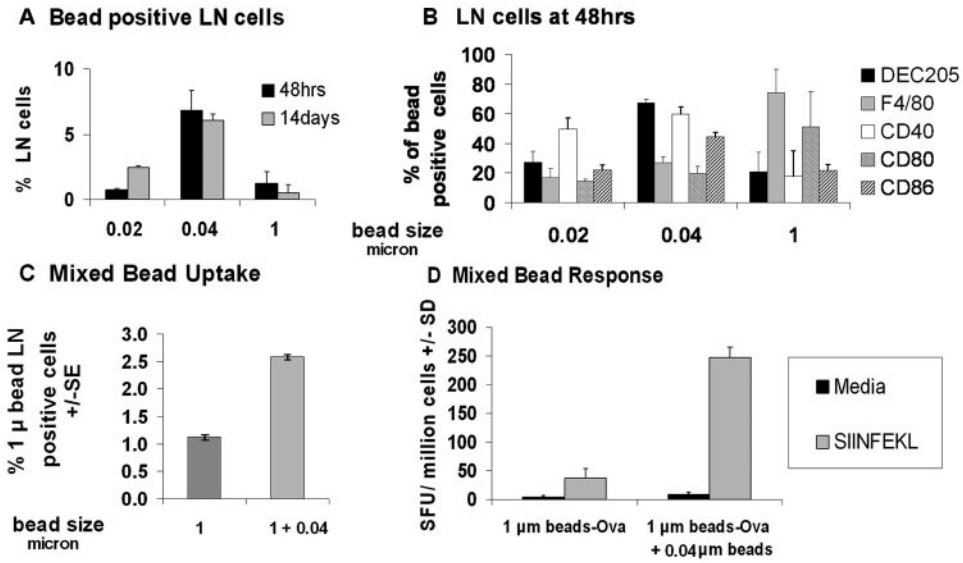

FIGURE 3. Localization of beads to draining LN. *A*, C57BL/6 mice were immunized intradermally in the footpad with 100 μg of OVA conjugated to 0.02-, 0.04-, or 1-μm fluorescent (FITC) beads, and the draining popliteal LN were dissected 48 and 14 days after immunization. The data are presented as the mean FITC percentage of positive LN cells $\pm$ SE. The percentage of uptake of 0.04-μm beads was significantly greater than uptake of the other bead sizes at both time points ($p < 0.01$). *B*, Phenotypic characterization of bead-positive cells at 48 h after injection of 0.02-, 0.04-, or 1-μm beads: the mean percentage $\pm$ SE for 3–14 mice tested for each marker is shown. Cells with 0.02-μm beads expressed more CD40 and less F4/80 than cells with 1-μm beads ($p < 0.05$ for both), while the cells with 0.04-μm beads expressed significantly more DEC205, CD40, and CD86 than cells with 1-μm beads ($p < 0.004$), and the latter expressed more F4/80 ($p = 0.003$) and CD80 ($p = 0.03$). *C*, Green fluorescent large 1-μm beads were injected alone (20 μl of 1% solids) or together with 0.04-μm red beads (10 μl of 1% solids of each) intradermally in the footpad of C57BL/6 mice and draining popliteal LN were dissected 24 h later for FACScan analysis. A total of 20,000–100,000 events was collected for each sample type. The data show the mean percentage $\pm$ SE of green fluorescent cells (positive for 1-μm beads) from the total LN cells from three animals per group. Enhanced numbers of 1-μm bead-positive cells (green) in the LN were found upon 0.04-μm bead coinjection ($p < 0.0005$). One of two similar experiments is shown. Similar enhanced 1-μm uptake was seen with unconjugated or OVA-conjugated particles. *D*, Spleen cells from mice immunized intradermally with 100 μg of OVA conjugated to 1-μm beads alone (1-μm beads-OVA) or mixed with unconjugated 0.04-μm beads (1-μm beads-OVA + 0.04-μm beads), as above, were tested after 9 days for the induction of IFN-γ-producing CD8 T cells by restimulation with SIINFEKL in ELISPOT assays. Enhanced induction of IFN-γ was found after coimmunization ($p < 0.005$).

unconjugated nano-beads also induced greater T cell responses compared with 1-μm OVA beads alone (Fig. 3*D*, $p < 0.005$).

Studies are ongoing, comparing different immunization routes, with results suggesting similar strong immunogenicity and abundant draining LN localization of nano-beads after s.c. administration in a different animal species. Analysis of cells in afferent lymph, draining the injection site in sheep, shows nano-bead-positive DC trafficking within 2 h from immunization (A. Gamvrellis, unpublished data).

Comparison with other adjuvants

The ability of nano-beads/OVA (0.04 μm) to induce CD8 T cell responses was compared with OVA administered in other adjuvants (QuilA, MPL, and Freund's), including the adjuvant licensed for human use, alum. To provide a comparison with a generally accepted potent CD8 T cell-inducing vaccine, DC were pulsed ex vivo with OVA (DC-OVA) or SIINFEKL (DC-peptide) and used to immunize mice. A comparison with both DC-SIINFEKL and DC-OVA was performed, with the latter providing a comparison with all conditions using the same immunogen. The soluble OVA pulsed to the DC needs processing through the right pathway to efficiently excise the SIINFEKL epitope and present it on MHC class I. The comparison with DC-SIINFEKL plays against the nano-beads, as they have to target OVA to be processed via the MHC class I pathway to generate SIINFEKL-MHC complexes on the surface as efficiently as coating DC with exogenous peptide. Fig. 4, *A* and *B*, shows that nano-beads were consistently the best adjuvant for induction of CD8 T cell responses with whole Ag (OVA), and superior (*A*, one dose) or comparable (*B*, two doses) to using the minimal SIINFEKL epitope pulsed directly onto live DC for immunization. In *A* and *B*, reactivity was assessed by IFN-γ production in response to the SIINFEKL epitope.

The ability of nano-beads to induce Abs was also compared. After two immunizations (Fig. 4*C*), nano-beads induced Ab titers comparable to CFA/IFA, the standard adjuvant for Ab induction in mice, and higher than all other adjuvants. Thus, the mean inverse Ab titers to OVA, induced by OVA-conjugated nano-beads (204,800 $\pm$ 58,055 SE, $n = 12$ animals), were significantly higher than those induced by QuilA, MPL, DC-OVA, alum, and DC-SIINFEKL ($p < 0.05$), and not significantly different from Freund's ($p = 0.47$). In all treated animals, vaccination with inert polystyrene nano-beads, via intradermal, s.c., and i.m. routes, failed to produce the local or peripheral inflammatory reactions observed with the other powerful adjuvants, Freund's and QuilA (data not shown). Ag conjugated to nano-beads was therefore very effective in stimulating both specific Ab and MHC class I-restricted T cell immunity.

Protection against tumor growth

EG7 OVA tumor model. C57BL/6 mice were immunized twice with 0.04-μm nano-beads/OVA intradermally at -28 and -14 days, then challenged at day 0 with EL4 thymoma cells transfected with cytoplasmic OVA (EG7 tumor cells) (Fig. 5, *A* and *B*). None (0 of 10) of the immunized mice developed EG7 tumors until >10 days (Fig. 5*B*), whereas tumors developed in 10 of 10 of the untreated naive controls by day 10 (Fig. 5*A*). The same pattern of protection was observed after a single immunization with 0.04-μm nano-beads/OVA (data not shown). Tumors that grew at >15 days

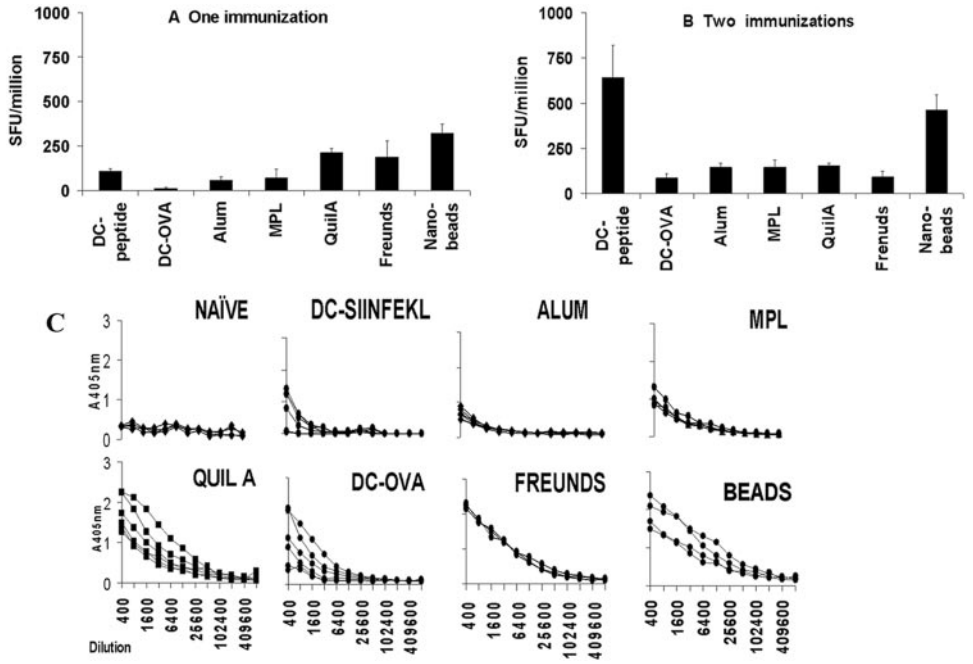

FIGURE 4. Comparison of nano-beads with other adjuvants. Comparison of MHC class I-restricted T cell responses: C57BL/6 animals were immunized intradermally with 100 μg of OVA with each of the adjuvants, either once (*A*) or twice (*B*) 14 days apart. Ten days after the last immunization, the number of SIINFEKL-specific T cells induced above background was assessed by IFN-γ ELISPOT. Mean SFU per million cells $\pm$ SE for each group is shown. The numbers of mice per group were: DC-peptide (one, $n = 3$; two, $n = 5$); DC-OVA (one, $n = 3$; two, $n = 4$); alum (one, $n = 3$; two, $n = 5$); MPL (one, $n = 3$; two, $n = 4$); QuilA (one, $n = 3$; two, $n = 5$); Freund's (one, $n = 8$; two, $n = 4$); nano-beads (one, $n = 6$; two, $n = 4$). The response for nanobeads after one immunization was significantly greater ($p < 0.025$) than responses induced by adjuvants other than QuilA and Freund's ($p > 0.1$). After two immunizations, nano-bead responses were significantly greater ($p < 0.02$) than all immunizations, except DC-peptide ($p > 0.1$). *C*, Comparison of Ab titers. Mice were immunized twice, as above. The numbers of mice per group were: naive ($n = 4$); DC-SIINFEKL ($n = 6$); alum ($n = 6$); MPL ($n = 5$); DC-OVA ($n = 6$); QuilA ($n = 6$); Freund's ($n = 3$); nano-beads ($n = 4$). One of three similar experiments is shown. Serum was collected 10 days after the second immunization, and OVA-specific IgG titers were determined by ELISA. The mean of the inverse titer for nano-beads was significantly higher than for all adjuvants tested ($p < 0.02$), except Freund's, which was comparable.

in immunized and some untreated groups were escape variants that had lost the ability to activate a T cell hybridoma specific for $H2^b$/SIINFEKL (13).

In side-by-side comparisons, other bead sizes conjugated to OVA were less effective. Comparison of the protective effects of 0.04- and 1-μm OVA-conjugated beads is shown at day 10 after tumor challenge. At this time point, tumors in naive mice reached maximum size, eight of eight mice immunized with 0.04-μm beads/OVA were protected (Fig. 5*C*), and therapy was effective (Fig. 6). After this time, late outgrowth of tumors was seen, and in other studies these were shown to be escape variants (T. Fifis and M. Plebanski, unpublished data). Thus, at day 10, mean tumor size in the 0.04-μm beads/OVA-vaccinated group was significantly less compared with mice immunized with 1-μm beads/OVA ($p < 0.05$) or soluble OVA ($p < 0.05$) (Fig. 5*C*). These data were confirmed by comparing total tumor size up to day 10, in which tumor growth in mice treated with soluble OVA was 73% of untreated controls; for 1-μm beads/OVA, it was 68%; and for 0.04-μm beads/OVA-vaccinated group, total growth was 20%, showing tumor growth inhibition in this group.

E7-HPV tumor model. In a second tumor model using the same EL4 thymoma, but now transfected with the E7 protein from HPV variant 16, it was possible to induce complete protection by immunization with nano-beads conjugated to a single E7 CD8 T cell epitope, E7.1 (Fig. 5*E*). When mice were challenged 30 days after immunization with 5×10^6 EL4-E7-transfected cells, all mice grew tumors (Fig. 5, *D* and *E*), but all of the nine mice in the nano-beads-E7.1 CD8-immunized group cleared the tumors by day 12 (Fig. 5*E*), whereas no mice in the naive group (data not shown) and only one mouse in the group immunized with E7.1 CD8 soluble peptide (Fig. 5*D*) were clear of tumors at this time. Tumors progressed in all but one of the latter group by day 21, and in all of the naive group. Thus, peptide alone failed to induce protection, while animals treated with beads-E7.1 peptide vaccine remained tumor free with no detectable escape variants when followed for up to 60 days (data shown to day 21).

Treatment of established tumors

With a view to a potential therapeutic use for such vaccines, mice with solid EG7 tumors at 8 days after tumor injection were divided into two groups: one was left untreated (Fig. 6*A*; naive, size range at day 8 was 6–70 mm^2); the other (Fig. 6*B*; size range 21–71 mm^2) was immunized with 0.04-μm nano-beads conjugated with OVA. By 19 days (11 days after a single vaccination), the mice in the immunized group had completely cleared their tumors, regardless of the initial solid tumor burden, whereas in six of six untreated mice tumors remained and were progressing in four of six mice.

Thus, a single administration of nano-beads conjugated to Ag has the potential to both protect from growth of new tumors and eradicate existing large tumors expressing a specific Ag in the OVA-EG7 model.

Discussion

The present study shows that Ag covalently coupled to virus-sized, solid core, inert nano-beads provided a potent immunogen for the induction of combined cellular and humoral responses. The nano-beads are novel in being sharply defined by the parameter of size,

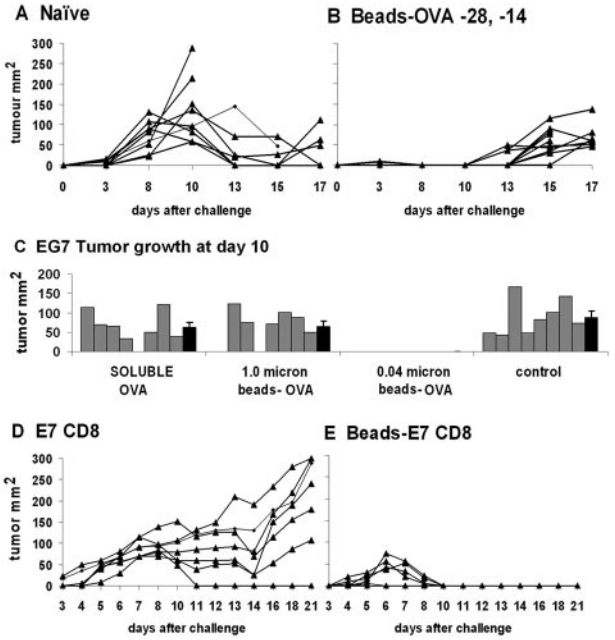

FIGURE 5. Protection against tumor challenge in the OVA-EG7 and HPV/E7-EL4 models. *A* and *B*, C57BL/6 mice were immunized intradermally with 100 μg of OVA conjugated to 0.04-μm beads at -28 and -14 days or left untreated (naive). All mice were challenged with OVA-expressing EL4 (EG7) cells s.c. at day 0, and tumor growth was monitored to day 17. Individual tumor growth curves for 10 animals in each group are presented. The difference in the frequency of tumors at all time points to day 10 (days 3, 7, and 10 after challenge) was highly significant ($p = 0.00001$). *C*, Protection was also seen 30 days after a single immunization with 0.04-μm beads/OVA (100 μg), with 8 of 8 mice tumor free at day 10 (tumor size, $p < 0.0001$ compared with controls; 0 of 8 tumor free), in contrast to mice immunized with the same dose of soluble OVA (0 of 8 tumor free; tumor size $p > 0.05$ compared with controls) or OVA conjugated to 1-μm beads (1 of 8 tumor free; tumor size $p > 0.05$ compared with controls). Individual tumor sizes are shown at day 10 for each group (▨), with mean tumor size ± SD included (■). *D* and *E*, Show protective immunity after tumor challenge with EL4 cells transfected with the HPV E7.1 peptide. C57BL/6 mice ($n = 7$–9/group) were: *D*, immunized once intradermally with soluble E7.1 CD8 peptide (100 μg dose), or *E*, immunized with conjugated nano-bead-E7.1 CD8 epitope (100 μg dose). At 30 days, mice were challenged with EL4-E7 tumor cells (5×10^6 EL4-E7.1 cells s.c.), and tumor growth was monitored for the next 21 days. Data are presented as individual tumor growth curves. The difference in tumor growth between the two groups was significant ($p < 0.05$).

and in providing no added known danger signals to induce immune activation. Nano-beads, after a single administration, induced reliable protection against a tumor expressing a model Ag, and were effective as a curative treatment for large, well-established tumors.

The ability of nano-beads to induce powerful and broad-spectrum immunity was due to a unique combination of bead size and covalent Ag conjugation. The size promoted efficient localization to $DEC205^+$ $CD40^+CD86^+$ cells in the draining LN. These cells are a DC/APC subset found primarily in s.c. areas, and are particularly efficient at presenting extracellular Ag (cross-priming) on MHC class I to CD8 T cells (14). Nano-beads do not incorporate known danger signals, such as bacterial components, associated with maturation and activation of DC, yet these beads localized to mature and activated DC phenotypes in the draining LN (4, 12, 15). We speculate that the danger signal may be built into the nano-beads by virtue of their size, and DC evolved to react appropriately to size clues within this specific viral size range. Interestingly, the optimal immunogenic size identified in our study

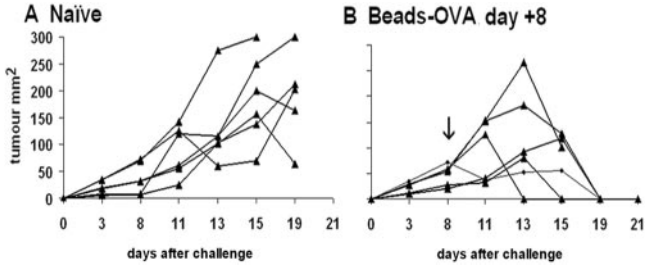

FIGURE 6. For therapy of established OVA-EG7 tumors (*A* and *B*), C57BL/6 mice were injected with EG7 cells s.c. on day 0, and tumor growth was monitored to day 8. Two groups of mice bearing similar tumor sizes were selected on day 8, one group (*A*) was left untreated (Naive), and the other (*B*) immunized intradermally once with 100 μg of OVA/0.04-μm beads. Individual tumor growth curves for six animals in each group after immunization are shown. All animals in the untreated group had substantial tumors at day 19. All animals in the immunized group were healthy and without evidence of tumors at days 19 and 21. The difference in the frequency of regressed tumors between the two groups was significant ($p = 0.04$).

for solid core nano-beads falls within the range observed for a variety of Flaviviruses, Arboviruses, Orbiviruses, and Reoviruses (0.03–0.08 μm).

Covalent linkage of Ag and nano-beads promoted vaccine stability, which may have contributed to immunogenicity and sustained immunity. In direct comparisons, nano-beads induced Ab levels equivalent to Freund's, CD8 T cells comparable to peptide-pulsed DC, and responses superior to a range of common adjuvants. We also tested protective and therapeutic efficiency of nano-vaccine conjugates targeting a model tumor Ag (OVA). A known tumor-associated Ag, HPV protein E7, also showed good protection results (16). Therapeutic immunization in the HPV-E7.1 model was not attempted. We are currently developing models in which low levels of tumor-associated Ags are expressed and there is no spontaneous regression in nonimmunized mice, providing a stringent test (17).

Preliminary experiments indicate the nano-bead approach can also induce protection against fully syngeneic untransfected melanomas (18).

Comparing our results with published vaccine literature on various Ags and vaccine techniques also indicates that 0.04-μm nano-bead conjugates induce immunity equal or superior to DC targeting, Prime-boost, or particle-based immunizations (3, 5, 6, 19, 20). This sized bead also has a unique ability to elicit combined humoral/cellular immunity and protection/clearance of tumors after a single dose. Other particulate vaccine formulations, such as VLP and ISCOMS that fall within a similar particle size range (0.02–0.06 μm), may act through different mechanisms to solid core nano-beads. The VLP MHC class I processing/presentation pathway is TAP independent (9, 21), while the nano-particles of the present study did not induce Ag-specific CD8 T cells in $TAP^{-/-}$ animals ($n = 3$, data not shown), suggesting a different fate in DC. Additionally, VLP may have a short $t_{1/2}$ in vivo, because they can be unstable when used with adjuvants or upon environmental changes (e.g., temperature), and include irrelevant and possibly competing antigenic determinants. ISCOMS have lipids able to fuse with cell membranes, but not necessarily those of APC (22). In this context, it is important to note that DC are particularly efficient at diversion of endosomal/phagosomal (23) Ags for MHC class I presentation (24, 25), and by patocytosis, an uptake mechanism for some hydrophobic nano-particles with the potential to deliver Ags into the cytosol (17, 26). Studies are in progress (beyond the scope of the present manuscript) to elucidate the uptake and processing mechanisms for nano-bead-conjugated Ag.

High potency in inducing protective immune responses and simplicity in vaccine formulation makes the concept of covalently conjugated Ag to inert carrier nano-beads useful for targeting whole recombinant Ags and mixtures of proteins containing multiple epitopes for MHC class I presentation and/or Ab induction: a useful experimental tool and potentially useful feature for vaccine use in MHC diverse human populations.

Acknowledgments

We thank Violeta Bogdanoska, Carla Osinski, Karen Scalzo, and Simone Gloster for technical assistance and comments, and Dr. Brigitta Askonas, Dr. Eli Sercarz, and Dr. Mauro Sandrin for comments on the manuscript.

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