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Research Paper

Poly (Lactide-co-Glycolide) Microspheres in Respirable Sizes Enhance an *In Vitro* T Cell Response to Recombinant *Mycobacterium tuberculosis* Antigen 85B

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Purpose. To investigate the use of poly (lactide-co-glycolide) (PLGA) microparticles in respirable sizes as carriers for Antigen 85B (Ag85B), a secreted protein of *Mycobacterium tuberculosis*, with the ultimate goal of employing them in pulmonary delivery of tuberculosis vaccine.
Materials and Methods. Recombinant Ag85B was expressed from two *Escherichia coli* strains and encapsulated by spray-drying in PLGA microspheres with/without adjuvants. These microspheres containing rAg85B were assessed for their ability to deliver antigen to macrophages for subsequent processing and presentation to the specific CD4 T-hybridoma cells DB-1. DB-1 cells recognize the $Ag85B_{97–112}$ epitope presented in the context of MHC class II and secrete IL-2 as the cytokine marker.
Results. Microspheres suitable for aerosol delivery to the lungs (3.4–4.3 μm median diameter) and targeting alveolar macrophages were manufactured. THP-1 macrophage-like cells exposed with PLGA-rAg85B microspheres induced the DB-1 cells to produce IL-2 at a level that was two orders of magnitude larger than the response elicited by soluble rAg85B. This formulation demonstrated extended epitope presentation.
Conclusions. PLGA microspheres in respirable sizes were effective in delivering rAg85B in an immunologically relevant manner to macrophages. These results are a foundation for further investigation into the potential use of PLGA particles for delivery of vaccines to prevent *M. tuberculosis* infection.

KEY WORDS: aerosol; Ag85B; macrophages; microspheres; *Mycobacterium tuberculosis*.

INTRODUCTION

Currently, the only tuberculosis (TB) vaccine available for human use is the attenuated strain of *Mycobacterium bovis* termed Bacillus Calmette-Guerin (BCG). However, the protective effect of the BCG vaccine has been shown to be highly variable. Many *Mycobacterium tuberculosis* (*MTB*) components have been tested as potential TB vaccine candidates in the form of subunit antigen proteins (1–5), fusion proteins (6,7), and DNA vaccines (8). Culture filtrate proteins (proteins secreted by *MTB*) are one category of subunit antigens that have received significant attention. These proteins are among the first *MTB* molecules to interact with the host immune system during infection, which is a desirable feature for a vaccine antigen. Since CD4 and CD8 T cell responses are the critical immunological responses for control of *MTB* infection in animals and humans (9,10), mycobacterial antigens must be presented in the context of peptide-major histocompatability complex class I and II (MHC class I, II) to elicit effective T cell mediated immunity.

Antigen 85 (Ag85) complex proteins are the most abundant *MTB* secreted proteins, accounting for as much as 41% of culture filtrate proteins (11). The complex consists of three subunit components: antigen 85A, B and C that share very high sequence identity to each other (68–79%) (12). This complex interacts with the immune system at an early stage of the infection process and induces both humoral and cell-mediated immune responses in *MTB*-infected patients (13,14). Significant effort has been expended to develop the Ag85 complex into a protective vaccine for tuberculosis. There has been some success with DNA vaccines of Ag85A

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(15,16) and Ag85B (17,18). A recombinant BCG strain overexpressing *MTB* Ag85B elicited a higher level of protection than traditional BCG (19). An attenuated *Listeria monocytogenes* strain carrying plasmids encoding the Ag85 complex was capable of inducing protein derivative-specific cellular immune responses (20). A fusion protein Ag85B-ESAT-6 (21) was reported to elicit strong immune responses and protective effects against *MTB*. The published results demonstrate that Ag85 complex or Ag85A and Ag85B molecules are among the most promising candidates for future TB vaccines.

Ag85B is the most abundant subunit among the three Ag85 complex components (22) produced during *MTB* growth, either during the culture of the bacteria in broth culture or intracellularly in human mononuclear phagocytes (23). Ag85B alone is immunoprotective in the highly relevant guinea pig model of pulmonary tuberculosis (24). While Ag85 complex and subunits can be isolated from culture medium, recombinant protein technology facilitates the production of Ag85B in quantities suitable for vaccine investigation (22,25).

There has been little investigation of alternative delivery systems and administration routes for vaccines to elicit effective immunity against *MTB*. Particulates have been used to improve vaccine antigen delivery. Microspheres of poly (lactic-co-glycolic acid) (PLGA) copolymers have been a major focus as carriers for antigens (26–29). These PLGA microsphere systems, which have diameters ranging from 1–10 μm, can interact with antigen presenting cells (APC) and antigens can be presented by APCs to specific T cells to induce cell-mediated immunity (CMI) (30,31). PLGA or PLGA-associated antigen particles enter the phagosome/phagolysosome and there is potential for some antigen to traffic to the cytoplasm (32). Moreover, PLGA microspheres have the ability to elicit CTL responses and the potential for mucosal immunization (33,34). Macrophages pulsed with antigens encapsulated in small particles can present antigen 100- to 1,000-fold more efficiently than macrophages pulsed with soluble antigen (35).

Since most TB patients acquire their primary infection via the pulmonary route, lung macrophages, which are the initial host cells for *MTB*, represent a logical site for vaccine delivery. Microspheres in the size range 1–5 μm have been delivered as aerosols to the lungs (36), and particles can reside at the site of deposition for extended periods of time prior to uptake by APCs (37). The targets of microsphere delivery are macrophages, which are mobile and number almost a billion in the periphery of the lungs (38). Continuous or pulsatile release of antigens from PLGA has been shown to provide a prolonged immunological response in animals and avoids the need for multiple boosting (39,40). Given the efficient targeting of microspheres to APCs, the particles have the ability to elicit strong immune responses even with small amounts of antigen (35).

In the present studies, the potential of PLGA microspheres to effectively deliver rAg85B to macrophages was evaluated. Subsequently, the ability of macrophages to process and present the effective epitope to antigen specific CD4 T-hybridoma cells (DB-1 cells) was measured by T cell production of IL-2, a marker cytokine of CMI. Production of two recombinant Ag85B (rAg85B) proteins and their encapsulation in PLGA microspheres suitable for pulmonary delivery are described. Macrophages pulsed with PLGA microspheres encapsulating rAg85B (PLGA-rAg85B) were shown to induce 92–360 fold greater antigen specific T-hybridoma cell response than soluble rAg85B alone. The longer lasting presentation of epitopes on the macrophages pulsed with PLGA-rAg85B resulted in an extended response compared to that of soluble antigen. The importance of surface-associated rAg85B on PLGA microspheres in eliciting an IL-2 response by the T-hybridoma cells will be illustrated. Our data show that microparticles encapsulating antigen can serve as an effective carrier for antigen delivery.

MATERIALS AND METHODS

Recombinant Antigen 85B. rAg85B protein was produced from two *E. coli* strains. The first *E. coli* strain was *JM109DE3*, containing Ag85B gene with His tag (courtesy of Dr Douglas Kernodle, Vanderbilt University). 1L Luria-Bertani (LB) broth with 50 μg/ml carbenicillin was inoculated with 20 ml of an overnight culture and grown at 37°C until the OD_{600} reading reached 0.4–0.5. The culture was induced by isopropyl-β-thiogalactopyranoside (IPTG) and grown at 22°C overnight. The *E. coli* cells were pelleted and then probe-sonicated. The supernatant was passed through a nickel-affinity column (Ni Sepharose™ 6 Fast Flow, Amersham Biosciences, Piscataway, NJ). The eluted fractions with His-tag proteins were further purified by Superdex 75 peptide column (Amersham Biosciences, Piscataway, NJ) with 20 mM Tris, 1M sodium chloride, pH 7.5 as the eluting buffer. The rAg85B was quantified at UV 280 nm with extinction coefficient of UV_{280} of 1.0 for a 1.0 mg/ml protein solution (41). The rAg85B purified from *JM109DE3* is referred to as rAg85B JM.

The second *E. coli* strain was *Origami B* strain carrying mutated thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes and a chaperone GroEL /GroES vector pGro7. The plasmid vector *pRSETB* was introduced into *Origami B* cells by a standard transformation protocol. Transformants were selected on agar plates with four antibiotics: carbenicillin, tetracycline, chloramphenicol and kanamycin. 1L LB broth and 50 μg/ml carbenicillin, 12.5 μg/ml tetracycline, 15 μg/ml kanamycin and 20 μg/ml chloramphenicol were inoculated with 20 ml of an overnight *Origami B* strain and grown at 37°C to reach OD_{600} reading 0.4–0.5. The culture was induced by 1 mg/ml L-arabinose and 0.2 mM IPTG and shaken at 22°C for 20 h. The remaining steps of the protein purification procedure were performed as described above. rAg85B purified from *Origami B* is referred to as rAg85B Chap.

Endotoxin in the protein preparations was removed by Detoxin-Gel™ Endotoxin Removing Gel (Pierce, Rockford, IL). The endotoxin level was detected by QCL-Chromogenic LAL (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) and was < 0.025 ng/mg after purification. The proteins were dialyzed in 0.1M ammonium bicarbonate and then lyophilized for 48 h.

SDS-PAGE. A precast 12% SDS-PAGE gel (Biorad, Hercules, CA) was used to determine the purity of the recombinant proteins. The gel was stained with Coomassie blue.

Cells and media. THP-1 cells (American Type Culture Collection) were maintained in RPMI 1640 (Invitrogen Corp., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 10 mM HEPES buffer, nonessential amino acids, 1% of antibiotics/ antimycotics (Invitrogen Corp., Grand Island, NY) which contain 100U/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B. The CD4 T-hybridoma DB1 cells (kindly provided by Dr W. Henry Boom, Case Western Reserve University) were derived from transgenic mice with human MHC genes for HLA-DR1. They are restricted to human HLA alleles and respond to human MHC molecules, which present $Ag85B_{97–112}$ epitope (42). DB-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Grand Island, NY) supplemented as indicated above (complete DMEM). Infection medium was DMEM supplemented with 10% non-heat-inactivated FBS with antibiotics/ antimycotics.

T-hybridoma Cell Recognition Assay. The anitigenicity of rAg85B was evaluated by a modified CD4 T cell hybridoma recognition assay (43). THP-1 cells were incubated in 96-well flat-bottom plates (1.5×10^5 cells/well) with 10ng/ml of phorbol myristate acetate (PMA, Sigma, St Louis, MO) in infection medium for 24 h to promote adherence to plates. Cells were washed once with infection medium and incubated with 100U/ml of recombinant human IFN-γ (Endogen, Woburn, MA) for 24 h. The cells were washed twice with infection medium prior to Ag exposure and were exposed to 100 μl of rAg85B at various concentrations or 100 μl of microsphere suspension in infection medium at 250 μg/ml. DB1 T-hybridoma cells (43) (10^5 cells/well, 100 μl, specific for recognition of $Ag85B_{97–112}$) were added into wells at the same time as antigens. The cells were co-incubated at 37°C for 24 h and supernatants were harvested. ELISA was used to measure the amount of IL-2 produced by T-hybridoma cells. (Biosource, Camarillo, CA).

Microsphere Preparation. PLGA polymer (700 mg)(MW 84.7 kd, L:G 75:25, intrinsic viscosity 0.68 dL/g in chloroform, Durect Corp., Pelham, AL) was dissolved in 200ml methylene chloride. Either 1 or 2 mg rAg85B were dissolved in 2.4 ml of 20 mM sodium phosphate buffer, pH 7.4 with or without the adjuvants muramyl dipeptide (MDP, 3.5 mg) or trehalose dibehenate (TDB, 3.5mg, Sigma, St Louis, MO). The molecular structure and adjuvancy of these molecules are well documented (44,45). The aqueous and organic phases were probe-sonicated for three 10 s periods on an ice bath immediately prior to spray-drying. The microspheres were manufactured using a spray-dryer (Buchi Mini Spray-drier B-191, Buchi, Flawil, Switzerland). The optimized conditions were: feed pump setting 50%, aspirator 50%, nitrogen flow 600 l/h, atomization pressure 3.0 bar, inlet temperature 65°C, outlet 41–43°C. The size and morphology of microspheres were monitored by scanning electronic microscopy (SEM, Model 6300, JEOL, Peabody, NY, USA). Stubs were coated with gold-palladium alloy (150–250Å) using a sputter coater (Polaron 5200, Structure Probe Incorporated Supplies, West Chester, PA, USA). The coater was operated at 2.2 kV, 20 mV, 0.1 torr (argon) for 60 s.

Confocal Microscopy. PLGA-sodium fluorescein microspheres were prepared for confocal microscopy. Following PMA treatment, the THP-1 cells were incubated with five particles/cell for 24 h. Particles were visualized using a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope at excitation and emission wavelengths 488 and 524 nm, respectively.

PLGA Microsphere Release Profiles. Microspheres of 90 mg PLGA-rAg85B (0.14% w/w)-MDP (0.5% w/w) were put into 2 ml of 67 mM potassium phosphate buffer (pH 7.4) containing 0.1% Tween 80 solution, and shaken in 37°C water bath. A volume of 1.5 ml of suspension was centrifuged for 10 min (Beckman GS-15R centrifuge, rotor F2402H at 12,000 rpm, 4°C) and 1 ml of supernatant was removed at intervals for measurement of rAg85B and MDP content. The rest of the sample and 1 ml of fresh medium were added into the dissolution vial. Lowry's method was used for protein analysis of rAg85B and HPLC method for MDP quantification. HPLC conditions for MDP were: C18 reversed phase column (4.6×25 mm), mobile phase 98% 25 mM ammonium phosphate buffer, pH 7.0 and 2% methanol at a flow rate 1 ml/min and UV wavelength of 200 nm.

Determination of the role of surface-associated-rAg85B on PLGA microspheres. PLGA-rAg85B JM (0.28% w/w)-MDP (0.5% w/w) microspheres were placed in 67 mM potassium phosphate buffer, pH 7.4, and vortexed for 3 h to remove the surface-associated antigen and then centrifuged. The supernatant solution was separated into two aliquots. One aliquot was mixed with PLGA control microspheres and the other aliquot was used directly in the assay. The residual PLGA-rAg85B-MDP microspheres, after the initial burst, were dried and reconstituted in the fresh medium into suspension at the microsphere concentration of 250 μg/ml. THP-1 cells were exposed to 100 μl of each sample, which were then subjected to the T cell hybridoma recognition assay.

Six-day Antigen Presentation Assay. THP-1 cells were incubated in 96-well flat-bottom plates (1.5×10^5 cells/well) with 10 ng/ml of PMA in infection medium for 24 h. Cells were washed once with infection medium and incubated with 100 U/ml of recombinant human IFN- for 24 h. The cells were: rinsed twice with infection medium; pulsed with 200 μl rAg85B Chap (25 μg/ml) or 200 μl 83 μg/ml of microspheres encapsulating rAg85B Chap (0.14% w/w) and MDP (0.5% w/w) and further incubated for 6 h; at that time the cells were washed extensively with infection medium to remove the antigen solution and microspheres. 200 μl DB-1 cells at density of 1×10^6 cells/ml were added at each time point (from day 1 to 6) to the wells. After 24 h co-incubation, the supernatant was harvested for IL-2 ELISA assay.

Statistics. The student's *t*-test was used for analysis. The statistical significance was set at $p<0.05$.

RESULTS

Soluble Recombinant Ag85B Purified from *E. Coli* can be Presented by Human THP-1 Cells to an Antigen Specific CD4 T-Hybridoma Cell

To reduce the potential for protein misfolding and aggregation, IPTG induction was performed at 22°C. The preparation of rAg85B JM yielded 16 mg/L after purification and was the sole protein band visible in SDS-PAGE (Fig. 1a). A second preparation of antigen, rAg85B Chap, was purified

from the *E. coli* strain *Origami B*. This *Origami B* strain of *E. coli* carries mutated thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes and a chaperone GroEL /GroES vector which facilitate disulfide bond formation in the less reducing environment of the cyotsol and could improve folding of Ag85B. The yield of rAg85B Chap was 4.5 mg/L after purification. The mass of the purified rAg85B JM as determined by mass spectrometry (Nanospray-ESI-MS on the Applied Biosystems Q-star Pulsar mass spectrometer, Foster City, CA) was 34 kD.

The soluble rAg85B protein was tested in the T-hybridoma cell recognition assay involving the human monocytic cell line THP-1 and DB-1 T-hybridoma cells which specifically recognize MHC class II presented $Ag85B_{97–112}$ epitope. This assay system and the DB-1 hybridoma cells were developed by Gehring *et al.* (43). Upon recognition of MHC-II presented epitope, DB-1 cells produce IL-2, which can be measured by ELISA assay. Using this T-hybridoma cell recognition assay we were able to measure presentation of the specific Ag85B peptide-MHC Class II complex by APC to T-hybridoma cells. Both rAg85B preparations were taken up by THP-1 cells, processed, and presented to the DB-1 hybridoma cells as measured by IL-2 production (Fig. 1b). IL-2 production reached a plateau at a concentration of rAg85B 25–50 μg/ml level. Interestingly, the rAg85B Chap induced higher IL-2 secretion from T-hybridoma cells DB-1 than the rAg85B JM in the concentration range of 12.5–150 μg/ml tested.

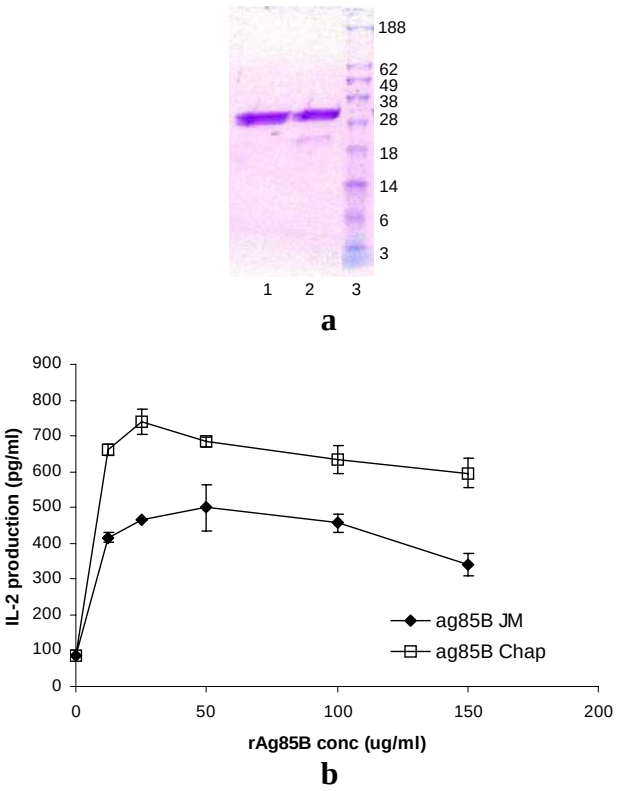

Fig. 1. a. SDS-PAGE of two recombinant antigen 85B. Lane 1, rAg85B JM; Lane 2, rAg85B Chap; Lane 3, molecular weight marker. **b**. Antigenicity of two recombinant Ag85Bs evaluated by T cell recognition assay. *Blank square* is rAg85B Chap, *solid diamond* is rAg85B JM.

PLGA Microspheres Encapsulating rAg85B (PLGA-rAg85B) can Present Antigen to DB-1 Hybridoma Cells

Thirteen batches of PLGA microspheres with various encapsulated components were manufactured under optimized conditions (Table I). The spray-dried microspheres were observed as raisin like structure showing some surface pores under the scanning electron microscopy (SEM). All the microspheres were in median size range from 3.4 to 4.3 μm with the geometric standard deviation range of 1.2–1.3 (Fig. 2a). This particle size is suitable for aerosol delivery (46). Using confocal microscopy we showed that similar sized PLGA-fluorescein microspheres were taken up by THP-1 cells after 24 h incubation (Fig. 2b).

Muramyl dipeptide (MDP) and trehalose dimycolate (TDM) are components of *MTB* cell wall. Trehalose dibehenate (TDB) is the synthetic analogue of TDM. The use of the cell wall components and their analgoues as adjuvants to stimulate the immune response is well suited to vaccine optimization. Control PLGA microspheres containing adjuvants only, PLGA-MDP, PLGA-TDB and PLGA-TDB-MDP formulations were added to THP-1s and coincubated with DB-1 cells. Similar IL-2 production was elicited by each of the above PLGA formulations in the T-hybridoma cell recognition assay and all responses were of the same order of magnitude (50–90 pg/ml). The addition of adjuvants alone to the PLGA microspheres did not produce large differences in IL-2 secretion with respect to the PLGA control particles. The PLGA particles themselves induced only a modest IL-2 response compared to the media only control, showing 80 *versus* 40 pg/ml respectively.

PLGA particles encapsulating rAg85B (0.14% w/w) and MDP (0.5% w/w) (PLGA-rAg85B-MDP) elicited significantly greater IL-2 secretion than soluble rAg85B or PLGA particle only controls. Soluble rAg85B solution alone at 36 ng dose/well (equivalent to microsphere dose) elicited a very weak IL-2 response of 79 pg/ml (± 2.1 SD). In comparison, the IL-2 released after PLGA-rAg85B-MDP exposure was 2,194 pg/ml (± 211 SD), which represented a 92-fold greater IL-2 response to PLGA-rAg85B-MDP microspheres than to the same amount of rAg85B in solution (after subtracting the medium control). Notably, the effect of PLGA-rAg85B-MDP on IL-2 production was dependent on encapsulation. Mixing PLGA-MDP (0.5% w/w) microspheres with 12.5 μg/ml soluble rAg85B (100 μl mixture for assay), which represented a larger amount of rAg85B than present in the PLGA-rAg85B-MDP microspheres used above, was also compared in the T cell hybridoma recognition assay to the encapsulated Ag85B particles. IL-2 secretion was approximately 170 pg/ml (± 14 SD), significantly lower than that observed with the PLGA-rAg85B-MDP (Fig. 3a). This demonstrated that mixing soluble rAg85B with PLGA-MDP elicited a significantly lower response (p=0.003). In conclusion, Fig. 3a shows that encapsulation of rAg85B into PLGA-MDP microspheres magnifies the antigenicity of rAg85B.

We also compared PLGA-MDP microsphere formulations encapsulating rAg85B JM and Chap in the hybridoma cell assay. IL-2 concentrations of 2194 pg/ml (± 211 SD) and 2,790

Table I. The 13 Batches of PLGA Microspheres were Manufactured Under Optimized Conditions

Batch of PLGA Microsphere	MDP (w/w Loading %)	TDB (w/w Loading %)	rAg85B JM (w/w Loading %)	rAg85B JM (w/w Loading %)
1	–	–	–	–
2	0.1	–	–	–
3	0.5	–	–	–
4	1.0	–	–	–
5	–	0.1	–	–
6	–	0.5	–	–
7	–	1.0	–	–
8	0.5	–	0.14	–
9	0.5	–	0.28	–
10	0.5	–	–	0.14
11	–	0.5	0.28	–
12	0.5	0.5	0.28	–
13	–	–	0.28	–

pg/ml (± 735 SD) were produced in response to PLGA-rAg85B JM-MDP and PLGA-rAg85B Chap-MDP, respectively (Fig. 3b). PLGA-microspheres containing rAg85B Chap gave a larger IL-2 response (360-fold after subtracting medium control) than the same amount of soluble rAg85B Chap that is consistent with observation of encapsulated rAg85B JM. There was no significant difference in IL-2 response between PLGA microsphere formulations encapsulating rAg85B JM or rAg85B Chap (p=0.249), even though soluble rAg85B JM and Chap elicited statistically different IL-2 responses in the concentration range of 12.5–150 μg/ml (p<0.001) (Fig. 1b).

The loading of rAg85B was increased two-fold in PLGA-MDP microspheres to 0.28% (w/w). The IL-2 response to rAg85B presented in these particles increased in comparison to those loaded with 0.14% reported above (Fig. 4a). PLGA-rAg85B JM (0.28% w/w)-MDP microspheres resulted in secretion of 4,532 pg/ml (± 216 SD) of IL-2, about a 1.24 fold increase in response to doubling rAg85B content. Testing an alternative adjuvant, PLGA-TDB microspheres containing rAg85B also elicited strong IL-2 secretion. PLGA-rAg85B (0.28% w/w)-MDP, PLGA-rAg85B (0.28% w/w)-TDB and PLGA-rAg85B (0.28% w/w) induced IL-2 responses of 4,532 (± 216 SD), 4,211 (± 146 SD) and 4,282 (± 165 SD) pg/ml, respectively (Fig. 4a). We also tested PLGA-rAg85B-TDB microspheres with added MDP, and these particles did not appear to increase the IL-2 responses in the T-hybridoma cell assay. However, the presence of rAg85B dramatically changed the response range of IL-2 secretion (Fig. 4b).

Interestingly, these results demonstrate that the presence of the adjuvants MDP or TDB in the microspheres in the concentration ranges tested did not influence the magnitude of response to rAg85B.

PLGA Microspheres Exhibited a Pulsatile Release Profile of rAg85B

The PLGA-rAg85B (0.14% w/w)-MDP (0.5% w/w) preparations provided a pulsatile release profile with high initial burst as demonstrated by *in vitro* dissolution (Fig. 5). In the first day 58% of the rAg85B was released from the microspheres. The cumulative quantity of rAg85B released

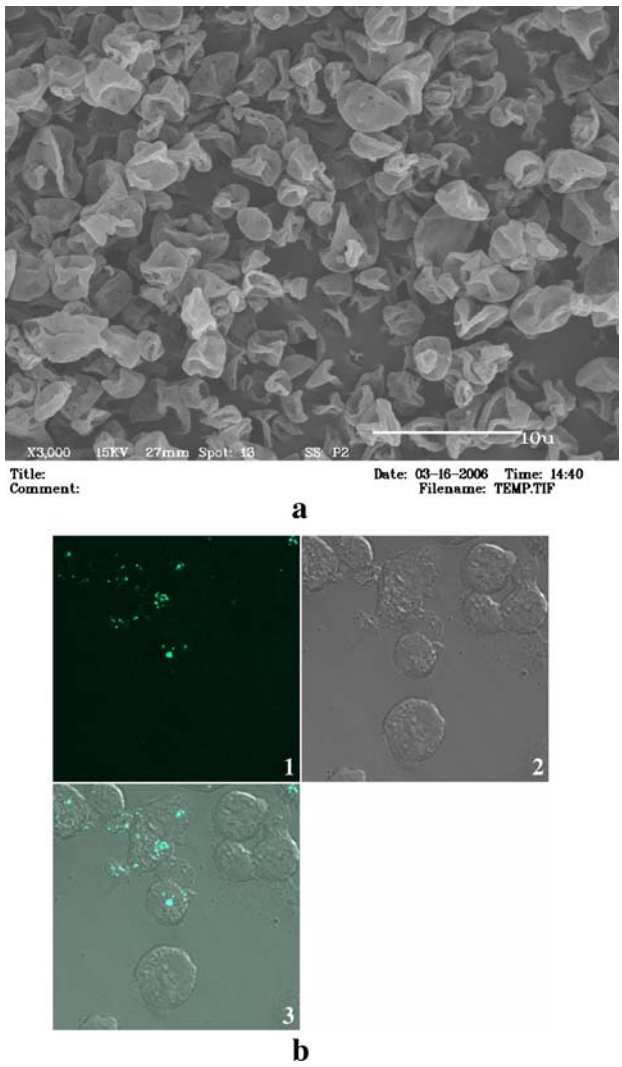

Fig. 2. a SEM of PLGA-MDP (0.5%)-rAg85B (0.14%) microspheres. The *standard bar* in the picture was 10 μm. **b** Confocal microscopy of phagocytosis of PLGA-sodium fluorescein particles by activated THP-1 cells. (1) Confocal image; (2) Normal image; (3) Overlap of the confocal and normal images.

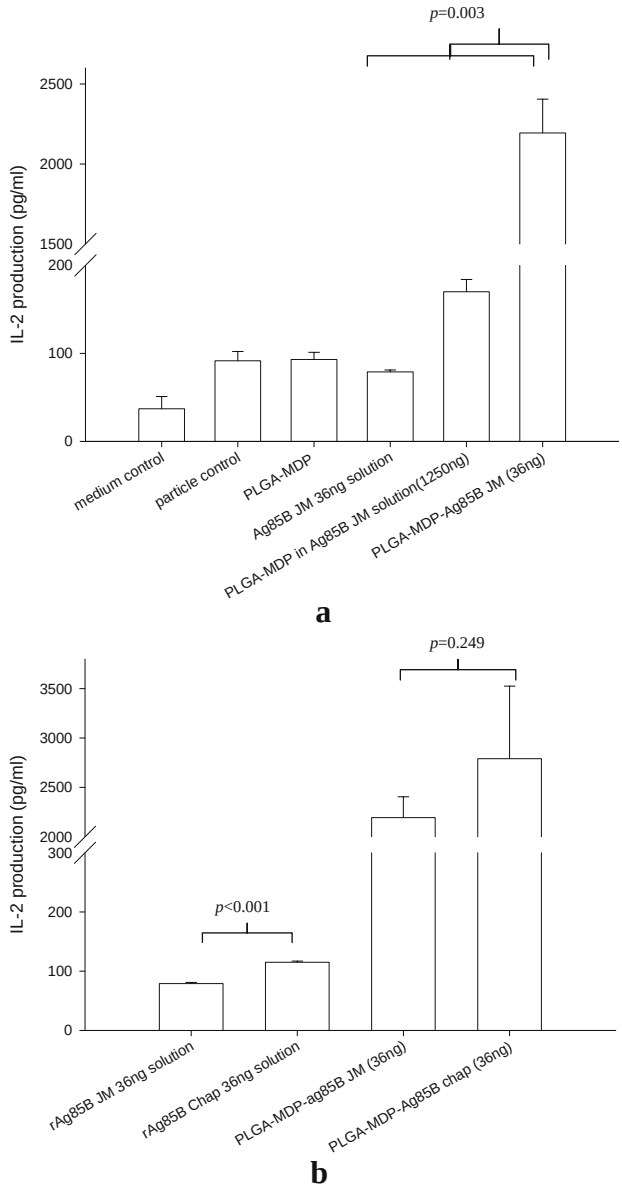

Fig. 3. T hybridoma cell response to different formulations, as measured by IL-2 ELISA. (n=3, *bar* is standard deviation in normal scale). All PLGA formulations contained MDP 0.5% loading and Ag85B as of 0.14% loading. **a**. The adjuvant effect of PLGA microspheres; **b**. The T hybridoma cell responses to Ag85B Chap and JM strains in different formulations. The p values were labeled in the figures.

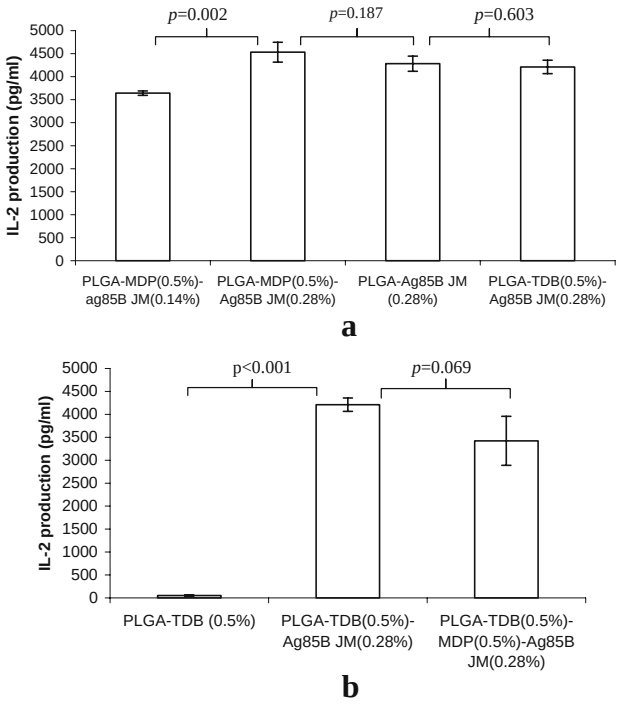

Fig. 4. T hybridoma cell response elicited by different doses and formulations of rAg85B in PLGA microspheres, as measured by IL-2 ELISA. (n=3, bar is standard deviation). **a**. Different doses of Ag85B JM in PLGA microspheres at loading of 0.14 and 0.28% and with/without the adjuvant MDP or TDB; **b**. Addition of MDP in the PLGA-TDB-Ag85B formulation did not improve the T hybridoma cell response.

reached a plateau of 66% on Day 3. Subsequently, a second period of release delivered an additional 14% (80% total release) by Day 20 and by Day 31, ~100% antigen was released. MDP was released from the microspheres in a large initial burst of 85% on day 1. The remaining 15% of MDP was continuously released over a period of 46 days. The initial burst reflects release of surface-associated protein and adjuvant.

The Importance of Surface-associated-rAg85B on PLGA-Microspheres

Surface-associated antigens and adjuvants were removed by washing and the residual microspheres were tested in the T-hybridoma cell recognition assay to determine whether the existence of surface-associated antigen was necessary for the antigen presentation activity of the formulation. The initial-burst solution, initial-burst solution plus PLGA control microspheres and residual dried-microspheres after initial burst, did not induced a response equivalent to the PLGA-rAg85B-MDP microspheres (Fig. 6). Therefore, surface-association of rAg85B appeared to be important for the T-hybridoma cell response to PLGA-rAg85B-MDP.

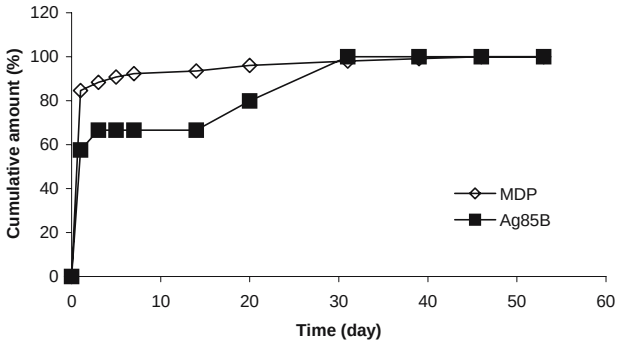

Fig. 5. Release profile of rAg85B JM and MDP from PLGA-rAg85B JM-MDP.

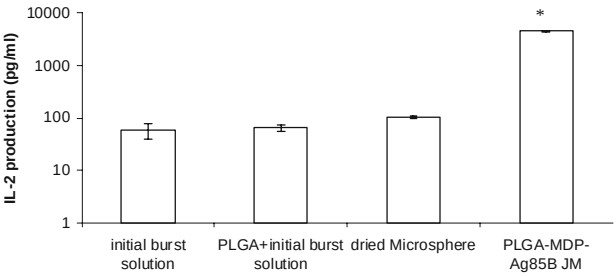

Fig. 6. The importance of surface-attached PLGA-rAg85B-MDP formulation on T hybridoma cell activation, measured by IL-2 ELISA. (n=3, *bar* is standard deviation). (* p<0.001).

PLGA Microspheres Encapsulating rAg85B Elicit Prolonged Epitope Presentation by THP-1 Cells

Prolonged epitope presentation by macrophages may be an advantageous property for a vaccine. The ability of PLGA microspheres containing rAg85B to present epitope over time was studied. Epitope presentation was compared over six days by THP-1 cells pulsed with soluble rAg85B Chap solution alone or the PLGA-rAg85B Chap (0.14% w/w)-MDP microspheres (Fig. 7). The absolute amount of IL-2 response differed, as shown in Fig. 3, for soluble antigen *versus* microspheres encapsulating antigen. By assaying T cell recognition daily we were able to study the epitope presentation of a given formulation on the initial Day 1 (Set at 100%) compared to subsequent Days 2 and 3, and the IL-2 response was unchanged. However, after Day three the level of IL-2 response fell. A more dramatic decrease in IL-2 response was observed with soluble antigen than with the PLGA-rAg85B-MDP microspheres. On Day six, 16% of the IL-2 response that was elicited on Day one was observed for soluble rAg85B, while 53% of the response observed with the microsphere formulation of rAg85B remained. Thus, the microsphere formulation of rAg85B not only amplified the response, but also provided longer-lasting epitope presentation. This prolonged presentation favors T cell recognition and the subsequent cytokine secretion.

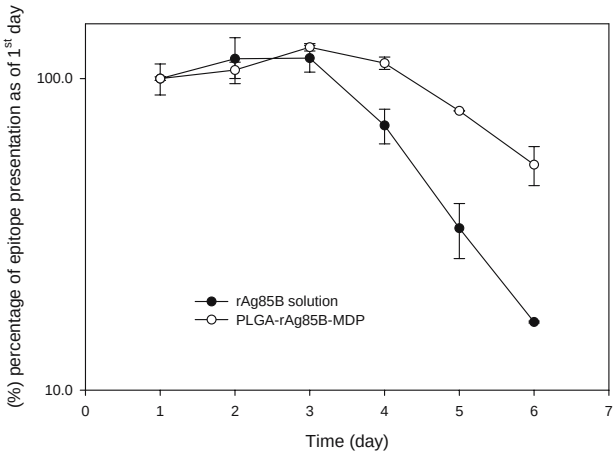

Fig. 7. Kinetics of epitope presentation by macrophages pulsed with rAg85B Chap solution or PLGA-rAg85B (0.14% w/w)-MDP. (n=3, *bar* is standard deviation).

DISCUSSION

PLGA microspheres encapsulating rAg85B delivered antigen to THP-1 cells for processing and presentation in the context of MHC class II to a CD4 T-hybridoma cell line DB-1. Microsphere formulations containing rAg85B were more effective in stimulating THP-1 cells to present antigen to the DB-1 T-hybridoma cells than rAg85B protein in solution. Submicrogram amounts of rAg85B in microspheres induced 92–360 fold larger IL-2 production than the same amount of soluble rAg85B. This effect was enhanced when the quantity of rAg85B incorporated into the microspheres was doubled.

Additionally, the two soluble rAg85B protein preparations used in this study differed in antigenicity based on T-hybridoma cell recognition assay. The rAg85B Chap preparation, purified from an *E. coli* strain carrying the mutated thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes and the chaperone GroEL /GroES vector elicited larger IL-2 production than rAg85B JM. Under physiological conditions, the *E. coli* cytoplasm is maintained in a reduced state that does not favor the formation of stable disulfide bonds in proteins. The *Origami B* mutations impair the reduction potential of both thioredoxins and glutathione (47). Furthermore, the chaperones provide a central compartment for a single protein chain to fold unimpaired by aggregation (48). Ag85B has one disulfide bond and one free −SH group. The purified rAg85B Chap may be structurally different from the rAg85B JM preparation, explaining the difference in antigen processing. Further investigation is required to define the difference between the different rAg85B proteins.

Subunit vaccines have the advantage of reducing side effects of possible infection that whole organisms may generate from vaccination. However, they frequently have weak antigenicity and immunogenicity and adjuvants are often needed for subunit antigen vaccination (49). PLGA microspheres have the depot effect associated with encapsulation of the antigens; therefore, microspheres themselves are adjuvants (35). Our data confirm this adjuvant effect compared to soluble antigen (Fig. 3). Addition of other adjuvants may further improve the antigenicity. However, the incorporation of adjuvants may also limit their use in humans. Furthermore, the use of adjuvants may be complicated by cross interaction of other naturally occurring adjuvants. For example, lipopolysaccharides can sensitize an animal's response to MDP (50,51). There was no statistically significant difference in IL-2 secretion between PLGA-rAg85B and PLGA-rAg85B-adjuvants at the concentrations employed in these studies. Therefore, the presence of adjuvant in our microsphere formulation was not necessary for the response elicited in this *in vitro* cell assay study.

Surfactants or emulsifiers were not used for the single emulsion microsphere preparation technique since they might influence phagocytosis (52–54). The large initial release of rAg85B from microparticles indicated that the single emulsion was not sufficiently stable for retention of internal water. However, the initial burst of rAg85B from the PLGA-subunit vaccine microsphere formulations was useful in subsequent studies, as outlined below. The subunit proteins are most likely associated with the microsphere surfaces by means of physical chain entanglement via

electrostatic and hydrophobic interaction (55). Proteins / peptides may be embedded in the microsphere matrix but some portion of peptide chains extend from the particulate surface. The initial release profile of rAg85B, which is different from MDP due to different size of molecules, may be explained by the extensive mixing, removing almost all of the rAg85B from the microsphere surface where diffusion from the microspheres would be the rate limiting phenomenon. A small quantity of protein would be left at the surface (56) given the low concentrations (< 1 μg/ml) and the high solubility of rAg85B. Following the initial release of rAg85B, the microspheres would behave as PLGA alone with respect to interaction with macrophages, until the remaining rAg85B was released from the microspheres by a mixture of diffusion and erosion mechanisms (57).

The microsphere formulation of rAg85B has been shown to initiate higher levels of T cell recognition as indicated by IL-2 production than soluble rAg85B, which is important for Th1 CMI. There are two possibilities why the PLGA-rAg85B formulation is better than soluble rAg85B. The first possibility is that exogenous soluble proteins can be taken up by endocytosis or fluid-phase pinocytosis. However, the uptake of rAg85B from solution is not as efficient as that from microsphere formulations. Macrophages are particularly sensitive to particles ranging from 1–10 μm. The phagocytosis of one microsphere with large quantities of rAg85B attached to the surface and within the polymer matrix, will result in higher concentration of the protein in the phagosomal compartment than endocytosis of a small number of rAg85B soluble molecules. Secondly, it is also possible that particulate antigens are processed differently from soluble antigens. Rock *et al.* reported that distinct epitopes from Ovalbumin were generated with differing efficiencies, by macrophages and B cells, from particles compared to soluble antigens. This may be due to changes in the structure of the bound antigen, conformation or its accessibility to proteases (58). The PLGA used in this study was a polymer of 75:25 lactide to glycolide. The generally hydrophobic surface exhibits hydrophilic regions which favor hydrophobic as well as electrostatic interaction with antigen proteins. Further studies are required to elucidate the effect of particle-antigen interaction on the epitope presentation.

Six-day epitope presentation data illustrated another potential advantage of microsphere formulation of vaccine antigens, that is, long-lasting epitope presentation that favors T cell recognition. In the first three days of the assay, the epitope presentation did not change compared to Day one. Antigen processing, forming complexes with MHC class II and sorting onto APC surface take some time, so there is concern over kinetic stability with respect to the complex of MHC class II: peptide epitopes. The kinetic stability of a MHC class II: peptide complex physiologically influences the recognition and expansion of specific T cells. Peptide epitopes must be loaded onto MHC class II in endocytic compartments and exported to the surface of APCs; this complex must be stable during the transit to the draining lymph node sustaining TCR signaling once contact between $CD4^+$ T cells and the antigen-bearing APCs (59). If $t_{1/2}$ of the complex is less than 5h, T cell immunity will not be generated *in vivo*. Immunodominant peptide complexes with MHC class II could possess extremely long half-lives of more than 150 h with MHC class II molecules (59). Ag85B peptide 91–108 is a dominant CD4 T cell recognition-epitope and has been recognized by 85% of healthy donors (60). It is inferred that the epitope Ag85B $_{97–112}$: MHC II complex must be sufficiently stable, although kinetic stability data is not available. On day six, the PLGA microsphere formulation of rAg85B still retained 54% of epitope presentation on APC surface, while soluble rAg85B had 16%. The release of rAg85B from microspheres increases the density of epitopes in phagosome (61) which favors the T cell recognition.

CONCLUSION

rAg85B is one of the most promising vaccine candidates with both class I and II epitopes. The ability of rAg85B in the microsphere systems to elicit CD4 T-hybridoma cell activation has been demonstrated in an *in vitro* T cell recognition assay. In conclusion, two expression systems were used to produce rAg85B. These antigens were incorporated at different concentrations into PLGA microspheres by a spray drying process. Microspheres delivered rAg85B more efficiently to macrophages than soluble antigen delivery. In turn, rAg85B can be processed and presented well at the macrophage surface in the context of MHC class II. PLGA-rAg85B microspheres stimulate an antigen specific CD4 T cell hybridoma response that is two orders of magnitude greater than that observed for soluble rAg85B. The PLGA-rAg85B particle effect can't be reproduced by mixing of soluble or initial burst solution with control PLGA particles. This suggests that the strong adjuvant effect of the particles requires surface absorption. These formulations provided extended epitope presentation on the APC surface for up to 6 days. This may mimic the boosting effects of independently administered vaccines but further studies are required to establish this proposition. Finally, the microspheres were prepared in a particle size range suitable for pulmonary delivery as aerosols. Taken together, this data demonstrates that dry powder delivery of rAg85B using a microsphere formulation has potential as a vaccine strategy for preventing TB and as a promising boosting vaccine.

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