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# Oral administration of polymer-grafted starch microparticles activates gut-associated lymphocytes and primes mice for a subsequent systemic antigen challenge

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*The mucosal and systemic humoral immune systems can function essentially independent of each other, responding to mucosal and parenteral antigens, respectively. Nevertheless, antigen administered by one route can modify responsiveness to subsequent immunization by an alternate route. Here we demonstrated, in mice, in addition to stimulating rapid and robust sera antibody responses, intragastric (i.g.) immunization with human serum albumin (HSA)-containing starch microparticles (MP) grafted with 3-(triethoxysilyl)-propyl-terminated polydimethylsiloxane (TS-PDMS) primed for enhanced specific sera IgG following a parenteral antigen boost. After as little as one i.g. immunization with microentrapped, but not with soluble, HSA antigen-specific proliferation and antibody secretion were detected in Peyer's patches (PP); this activity peaked after three i.g. MP immunizations. We observed a progressive dissemination of antigen-specific lymphocyte reactivity from PP to splenic tissue following oral MP immunization. Similarly, we observed a shift in HSA-specific antibody-secreting cells from PP and mesenteric lymph nodes to splenic tissue following i.g. MP immunization. We also demonstrated that oral immunization with microentrapped, but not with soluble HSA, resulted in enhanced numbers of spontaneous Th2-cytokine secreting lymphocytes which disseminated from mucosal to systemic lymphoid compartments. This observation coincided with our findings that HSA-specific sera IgG1 responses in animals given HSA in MP were significantly higher than those detected in the sera of mice given soluble HSA i.g., both before and after parenteral antigen challenge. These findings suggest that orally-administered TS-PDMS-grafted MP, by stimulating elements of the mucosal immune system, are a valuable addition to mucosal and systemic vaccine delivery systems.* © *1998 Elsevier Science Ltd. All rights reserved*

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Presently, most vaccines are administered via systemic routes and although parenteral immunization is effective in stimulating systemic immunity, specific mucosal immunity is not often induced. In contrast, mucosal immunization provokes both mucosal and systemic immunity via the stimulation of mucosally-situated IgA and IgG plasmacyte precursors[1,2]. These observations predict that mucosal, but not parenteral, immunization induces immune responses which are capable of both protecting the host from mucosal invasion and eliminating intruders from systemic tissues if the mucosae are breached. In concert, these mucosae-derived responses prevent or reduce the morbidity and mortality resulting from both mucosal and systemic infections[3].

We previously demonstrated that human serum albumin (HSA)-containing, biocompatible starch microparticles (MP) grafted with the silicone polymer 3-(triethoxysilyl)-propyl-terminated polydimethylsiloxane (TS-PDMS) stimulated both secretory and circulating antigen-specific humoral immunity following intragastric (i.g.) immunization[4]. Maximal sera HSA-specific responses occurred following oral boosting[4]. This response was manifested in the systemic compartment of the immune system and suggested that

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the initial immunization protocol could induce systemic humoral memory. Indeed, some reports suggest that feeding soluble antigens can sensitize animals for systemic DTH responses[5–7]. Additionally, since orally-induced memory cells have been repeatedly demonstrated to be activated following subsequent antigen challenge with cholera toxoid or its subunits[8–11], a potent mucosal and systemic antigen, i.g. priming of mucosal tissues with TS-PDMS-grafted MP might also lead to the generation of systemic memory capability, a phenomena not consistently seen with other particulate delivery systems[5].

In this study, we investigated whether orally-administered HSA-containing TS-PDMS-grafted MP primed mice for a systemic challenge with soluble antigen. The results showed that i.g. immunization with low doses of microentrapped, but not soluble, HSA activated lymphocytes in the gut-associated lymphoid tissue (GALT) and primed animals for significantly enhanced HSA-specific humoral immune responses following intraperitoneal (i.p.) HSA challenge. The results further support the use of TS-PDMS-grafted MP as a delivery vehicle for mucosal and systemic vaccines.

## MATERIALS AND METHODS

### Microparticle fabrication and characterization

HSA (Sigma, St. Louis, MO) was entrapped into TS-PDMS-grafted MP using an emulsion-based process and characterized as previously described[4]. Microparticles fabricated by this process routinely contained 5–6% w/w protein.

### Immunizations

Female BALB/c mice, age 6–8 weeks (Harlen Sprague Dawley Inc., Indianapolis, IN) were immunized i.g. on days 0, 7 and 14 with 50 $\mu$g of soluble or microentrapped HSA in 500 $\mu$l of 0.2 M $NaHCO_3$, or vehicle alone using PE50 tubing (Becton Dickinson Co., Mountain View, CA). In some experiments, animals were subsequently immunized intraperitoneally (i.p.) on day 19 with 100 $\mu$g HSA solubilized in phosphate-buffered saline (PBS, pH 7.4).

### Collection and preparation of sera and cells

Individual blood samples were obtained via the retro-orbital plexus. For sera evaluations, insoluble material was removed by centrifugation and sera were stored at −70°C until used.

Pooled spleens (SPL), mesenteric lymph nodes (MLN) and PP were isolated into ice cold Hanks' balanced salt solution (HBSS, pH 7.4). Single cell suspensions of SPL and MLN were prepared by crushing the tissues between the frosted ends of two microscope slides. Cell suspensions of PP were prepared as previously described[4]. Single cell SPL, MLN and PP suspensions were washed twice with HBSS by centrifugation, and the erythrocytes and dead cells were removed using Ficoll-Paque[12] (Pharmacia, Uppsala, Sweden). The remaining cells were resuspended in RPMI-1640 media supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine and 1% *N*-n-hydroxyethylpiperizine-N'-2 ethanesulfonic acid (HEPES), pH 7.4 (operationally termed RPMI-5). The viability of cell preparations routinely exceeded 90% as judged by ethidium bromide/fluorescein diacetate staining[13,14].

### Measurement of HSA-specific antibody responses

An enzyme-linked immunosorbant assay (ELISA) was used to measure HSA-specific antibody responses in individual sera as previously described[4]. Briefly, duplicate serial dilutions of sera were incubated on HSA-coated microtitre plate wells. HSA-specific antibodies were quantitated by incubating wells with heavy chain-specific, alkaline phosphatase-conjugated goat anti-mouse IgG, IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL). Responses were quantitated by measurement of optical densities (OD) at 405 nm following incubation of wells with 1.0 M diethanolamine buffer, pH 9.8, containing 50 mM $MgCl_2$ and 1.0 mg ml$^{-1}$ *p*-nitrophenylphosphate (5 mg phosphatase substrate tablets; Sigma). Sera prepared from buffer-treated animals were used to establish baseline OD values. The results were expressed as reciprocal end-point sera titres representing the greatest sera dilutions giving OD values exceeding two times buffer-alone mean values.

### Lymphocyte proliferative assay

Quadruplicate cultures of PP, MLN and SPL lymphocytes ($5 \times 10^5$) were prepared in 96-well, round-bottomed, sterile plates in the presence or absence of various amounts of HSA (250–1000 $\mu$g ml$^{-1}$) for 96 h at 37°C with 5% $CO_2$ in air. One $\mu$Ci of [$^3$H]thymidine ([$^3$H]Tdr, 740.0 GBq mmol$^{-1}$; Dupont/NEN, Mississauga, ON) was added to each well for the final 24 h of culture. Cells were harvested with a PhD Harvester (Cambridge, MA) and [$^3$H]Tdr incorporation was measured by standard liquid scintillation counting methods. The results were expressed as mean cpm from quadruplicate cultures.

### Enumerating of HSA-specific spot-forming cells

An enzyme-linked spot-forming assay (ELISPOT) was used to detect HSA-specific spot-forming cells (SFCs) in pooled PP, MLN and SPL preparations as previously described[4]. Duplicate serial dilutions of single cell suspensions (beginning at $1 \times 10^5$ per 100 $\mu$l per well) were examined using nitrocellulose plates (Millititer HA; Millipore Corp., Bedford, MA) incubated with 100 $\mu$l HSA (100 $\mu$g ml$^{-1}$ in PBS). The results represent the mean number of spots per $1 \times 10^6$ cells in duplicate wells containing at least twofold more spots than wells incubated with cells from buffer-treated animals.

### Enumeration of IFN-$\gamma$ and IL-4-producing cells

An ELISPOT assay was also used to quantitate spontaneous cytokine spontaneously released by MLN and SPL cells *in vitro*. Spontaneous cytokine secretion was examined since we concluded that by examining cytokine production immediately following lymphocyte isolation, and not after extensive *in vitro* manipulation, we would have a closer approximation of the *in vivo* cytokine microenvironment following i.g. immunization with microentrapped or soluble antigen. To detect