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Induction of mucosal and systemic immune responses by immunization with ovalbumin entrapped in poly(lactide-co-glycolide) microparticles

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SUMMARY

We have examined the range of mucosal and systemic immune responses induced by oral or parenteral immunization with ovalbumin (OVA) entrapped in poly(D,L-lactide-co-glycolide) (PLG) microparticles. A single subcutaneous immunization with OVA–PLG primed significant OVA-specific IgG and delayed-type hypersensitivity (DTH) responses. The DTH responses were of similar magnitude to those obtained using immunostimulating complexes (ISCOMS) as a potent control adjuvant, although ISCOMS stimulated higher serum IgG responses. Both vectors also primed OVA-specfic *in vitro* proliferative responses in draining lymph node cells following a single immunization and strong OVA-specific CTL responses were found after intraperitoneal (i.p.) immunization. ISCOMS were more efficient in inducing cytotoxic T lymphocytes (CTL), requiring much less antigen and only ISCOMS could stimulate primary OVA-specific CTL responses in the draining lymph nodes. Multiple oral immunizations with OVA in PLG microparticles or in ISCOMS resulted in OVA-specific CTL responses and again ISCOMS seemed more potent as fewer feeds were necessary. Lastly, multiple feeds of OVA in PLG microparticles generated significant OVA-specific intestinal IgA responses. This is the first demonstration that PLG microparticles can stimulate CTL responses *in vivo* and our results highlight their ability to prime a variety of systemic and mucosal immune responses which may be useful in future oral vaccine development.

INTRODUCTION

There is currently a great deal of interest in the development of synthetic vaccines containing recombinant proteins as protective antigens. However, purified proteins are poorly immunogenic and most available adjuvants are not effective with all immunogens and stimulate only humoral responses.[1,2] Protein antigens also do not stimulate the local immunity which is essential for protecting against diseases of mucosal surfaces such as the intestine and respiratory tract.[3] In addition, oral immunization is one of the few effective means of inducing vertical transmission of protection from mother to infant via breast milk. Thus there is a need for vectors of simple construction that will induce a full range of immune responses to orally administered protein antigens.

The poly(lactide-co-glycolides) (PLG) are polymers which form biodegradable microparticles into which antigens can be entrapped. These have been designed to release antigen over a predetermined period after a single immunization, thus abrogating the need for booster doses of vaccines.[4] A number of studies have indicated that entrapment of antigen into PLG microparticles generates enhanced antigen-specific immune responses.[5-7] Parenteral immunization with antigen entrapped in microparticles induces systemic IgG responses comparable to those elicited by other adjuvants, such as complete Freund's adjuvant (CFA)[5] or aluminium hydroxide, and this persists for a year after immunization.[6] In addition, it has been shown recently that PLG microparticles induce both salivary IgA and systemic IgG responses to entrapped ovalbumin (OVA) following repeated oral immunizations.[7] Hence, PLG microparticles appear to be promising vectors for the induction of mucosal and systemic immune responses when given orally.

The aims of the present study were to extend these findings by assessing the full range of immune responses induced by oral and parenteral immunization with PLG microparticles. In particular, we were interested to examine the efficiency of PLG-associated antigen to stimulate cell-mediated responses such as T-cell proliferation and cytotoxic T-lymphocyte (CTL) activity and to compare these with the responses obtained using

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Abbreviations: CFA, complete Freund's adjuvant; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; DTH, delayed-type hypersensitivity; E:T, effector:target; FCS, fetal calf serum; HPLC, high-performance liquid chromatography; i.p., intraperitoneal; ISCOMS, immunostimulating complexes; OVA, ovalbumin; PBS, phosphate-buffered saline; PLG, poly(D,L-lactide-co-glycolide); rhIL-2, recombinant human interleukin-2; s.c., subcutaneous.

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immunostimulating complexes (ISCOMS) which elicit powerful humoral and cell-mediated immune responses when administered either orally or parenterally.[8-11]

MATERIALS AND METHODS

Animals

Female BALB/c and C57BL/6 mice were obtained from Harlan Olac Ltd (Bicester, U.K.) and were first used when 6–10 weeks old.

Antigens

OVA (Grade V) and concanavalin A (Con A) were obtained from Sigma Chemical Co. (Poole, U.K.).

Preparation of OVA-containing microparticles

The microparticles were prepared using a solvent evaporation technique as described previously.[12] Briefly, a solution of PLG polymer (Resomer RG503, 50:50 lactide:glycolide ratio; Boehringer, Ingleheim, Germany) in dichloromethane [high-performance liquid chromatography (HPLC) Grade; May and Baker, Dagenham, U.K.] was emulsified with an OVA solution in double-distilled water using a Silverson homogenizer (Silverson Machines Ltd, Chesham, U.K.) to produce a water-in-oil emulsion. This was then added to a much larger volume of an aqueous solution of polyvinyl alcohol (PVA, 88% hydrolysed; Aldrich Chemical Co., Poole, U.K.) and homogenized to produce a stable water-in-oil-in-water emulsion, which was then stirred overnight at room temperature to allow solvent evaporation, with resultant microparticle formation.

Microparticles were collected by centrifugation, washed three times to remove non-entrapped OVA and freeze dried. The protein content was determined in a bicinchoninic acid assay (Sigma) after extraction of the protein as described previously.[13] Mean particle size as determined by photon correlation spectroscopy was 660 nm volume mean diameter (VMD).

Preparation of ISCOMS containing OVA

OVA was palmitified and incorporated into ISCOMS containing phosphatidyl choline, cholesterol and Quil A as described previously.[9,14] The formation of ISCOMS was confirmed by electron microscopy and after filter sterilization the ISCOMS were stored at -20° before use. The ISCOMS–OVA used in this study were 30–40 nm in diameter, incorporated >90% of the original protein as determined by Bradford's stain (BioRad, Hemel Hempstead, U.K.) and contained OVA and Quil A at a ratio of 10:1.[14]

Induction and measurement of immunity in vivo

Parenteral immunization. Systemic IgG and delayed-type hypersensitivity (DTH) responses were measured in BALB/c mice which were immunized in the rear footpad with either 100 μg OVA entrapped in PLG microparticles or 10 μg OVA in ISCOMS. Control mice received saline only. One week later the mice were bled from the retro-orbital plexus for determination of primary antibody levels and systemic DTH responses measured after a further week by challenging the opposite rear footpad with 100 μg heat-aggregated OVA. One week after challenge the mice were bled for determination of secondary antibody levels.

For the induction of antigen-specific proliferative responses *in vitro*, BALB/c mice were immunized in the rear footpad with either 50 μg OVA in microparticles, 5 μg OVA in ISCOMS, or saline only and the draining popliteal lymph node cells were isolated 14 days later.

Splenic CTL responses were induced by immunizing C57BL/6 mice with 100 μg OVA in microparticles, 5 μg OVA in ISCOMS, or saline intraperitoneally (i.p.) and the spleen cells were isolated 1 week later. To induce popliteal lymph node CTL responses, C57BL/6 mice received identical priming doses into the rear footpad and the draining popliteal lymph node cells were isolated 4 days later.

Oral immunization. Intestinal IgA responses were assayed in BALB/c mice which received six feeds of 100 μg OVA in PLG microparticles or saline only on days 1, 2, 3, 8, 9 and 10. Intestinal washes were performed 1 week after the last feed as described previously.[15]

Systemic CTL responses were measured in C57BL/6 mice which received three or six feeds of either 100 μg OVA in PLG microparticles, 50 μg OVA in ISCOMS, or saline and spleen cells were isolated 1 week after the last feed.

Measurement of OVA-specific antibody responses

Serum IgG and intestinal IgA antibody responses were measured using an enzyme-linked immunosorbent assay (ELISA) as described previously.[9,15] Briefly, OVA (10 μg/ml in 50 mM carbonate buffer) was adsorbed to microtitre plates (Immulon 4, Dynatech Laboratories Ltd, Billingshurst, U.K.) by overnight incubation at 4° and the plates were then washed three times with phosphate-buffered saline (PBS)/0·05% Tween-20 (BDH, Poole, U.K.) before addition of 100-μl samples. Sera were diluted 1:400 in PBS/Tween/1% normal rabbit serum before assay, whereas the intestinal washes were assayed neat. The samples were incubated for 2·5 hr at room temperature and the plates were washed three times with PBS/Tween before addition of 100 μl of a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG or goat anti-mouse IgA (both Sigma), respectively. After a further 3-hr incubation at room temperature, the plates were washed three times with PBS/Tween and 100 μl of phosphatase substrate (1 mg/ml; Sigma) was added to each well. After a 30–45-min incubation at room temperature, the optical density (OD) 405 nm of the samples was measured using a Dynatech MR5000 ELISA reader (Dynatech). Samples from individual mice were assayed in triplicate and expressed as OD 405 nm.

Measurement of systemic DTH responses

DTH responses were determined by measuring the increase in footpad thickness 24 hr after challenge of primed mice with 100 μg heat-aggregated OVA.

Measurement of antigen-specific proliferative responses

Two weeks after footpad immunization, draining popliteal lymph nodes were removed and single-cell suspensions prepared by teasing the nodes apart using needles, followed by gentle pipetting and passage through Nitex (Cadisch & Sons Ltd, London, U.K.) to remove any clumps or debris. After washing twice in RPMI-1640, viability was assessed using phase contrast microscopy and the lymph node cells were plated in quadruplicate in 96-well flat-bottomed microtitre plates (Costar, Northumbria Biologicals, Cramlington, U.K.) at $2·5 \times 10^5$ cells/well in

a total volume of 200 μl RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS) and penicillin, streptomycin, fungizone and glutamine (all Gibco BRL, Paisley, U.K.) and 5×10^{-5} M 2-mercaptoethanol (Sigma). The cells were cultured with various concentrations of OVA or Con A, at 37° in a humidified incubator containing 5% CO_2. After 3 days (Con A) or 6 days (OVA), cultures were pulsed with 1 μCi [^{3}H]thymidine/well for 16 hr before being harvested onto glass fibre filters using a 96-well harvester and read using a liquid scintillation counter (all Wallac, Turku, Finland).

Measurement of OVA-specific cytotoxic T-cell responses

OVA-specific CTL activity in the spleen was assayed using the OVA-transfected EG7.OVA cell line as described in detail elsewhere.[16] Briefly, spleen cells were isolated from immunized mice and restimulated *in vitro* for 5 days with mitomycin c-treated EG7.OVA cells. After restimulation, the effector cells were assayed for CTL activity by incubation at different effector:target (E:T) ratios in quadruplicate with ^{51}Cr-labelled EG7.OVA cells in a total volume of 200 μl V-bottomed microtitre plates (Costar) for 4 hr at 37°. One hundred microlitres of supernatant was then removed for analysis of ^{51}Cr release in a gamma-counter (Wallac). Non-OVA-expressing EL4 cells were used to control for non-specific lysis by effector cells.

CTL activity in popliteal lymph nodes was determined as described previously.[17] After isolation and washing, lymph node cells were cultured *in vitro* for 4 days with 10 U/ml recombinant human interleukin-2 (rhIL-2) (Cetus, Emeryville, CA) in RPMI-1640 containing 10% FCS, penicillin, streptomycin, fungizone, glutamine and 2-mercaptoethanol in 24-well tissue culture plates (Costar) at 2×10^6 cells/well at 37° in a humidified incubator containing 5% CO_2. After culture, the effector cells were assayed for CTL activity using 5×10^3 ^{51}Cr-labelled target cells per well. In all assays, the per cent OVA-specific CTL activity was calculated by the following formula:

$$\% \text{ specific lysis} = \frac{(\text{experimental c.p.m.} - \text{spontaneous c.p.m.})}{(\text{total c.p.m.} - \text{spontaneous c.p.m.})} \times 100\%$$

Spontaneous release was obtained using cells from saline-immunized mice which had been restimulated *in vitro* with EG7.OVA cells or rhIL-2 respectively, while total release was obtained using 10% Triton X-100 (Sigma).

Statistics

Results expressed as means ± standard deviations (SD) were compared by Student's *t*-test.

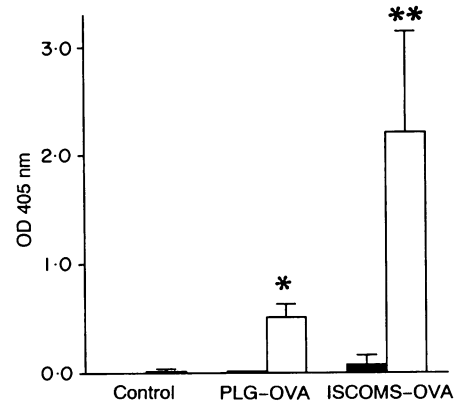

Figure 1. Priming of OVA-specific serum IgG responses by parenteral immunization with OVA in PLG microparticles or OVA in ISCOMS. (■) 1° responses were assayed 1 week after footpad immunization with either 100 μg OVA in PLG microparticles, 10 μg OVA in ISCOMS or saline only. (□) 2° responses were assayed 1 week after challenging these mice in the opposite footpad with 100 μg heat-aggregated OVA. Results shown are means ± 1 SD for six mice per group. *$P < 0.001$ versus control; **$P < 0.005$ versus PLG-OVA, $P < 0.001$ versus control.

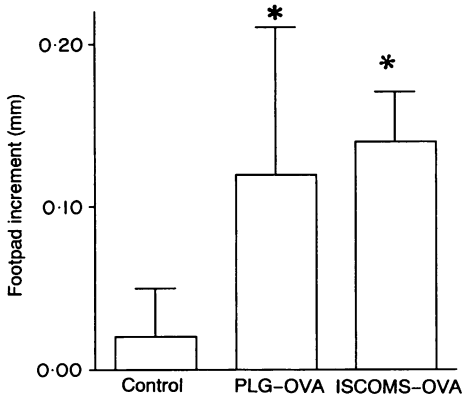

Figure 2. Priming of OVA-specific systemic DTH responses by parenteral immunization with OVA in PLG microparticles or OVA in ISCOMS. DTH responses were measured 24 hr after challenge with 100 mg heat-aggregated OVA into the rear footpad of mice primed 14 days previously in the opposite footpad with either 100 μg OVA in PLG microparticles, 10 μg OVA in ISCOMS or saline only. Results shown are means ± 1 SD for six mice per group. *$P < 0.05$ versus control.

RESULTS

OVA entrapped in PLG microparticles or in ISCOMS primes for OVA-specific IgG and DTH responses

Our first experiments set out to confirm the adjuvant activity of PLG microparticles for parenteral immunization and to compare them with ISCOMS. Virtually no primary IgG antibody response was observed in mice immunized into the rear footpad with either OVA in PLG microparticles or OVA in ISCOMS 7 days after immunization. However, both PLG microparticles containing OVA and ISCOMS–OVA primed mice to develop highly significant OVA-specific IgG responses after challenge with heat-aggregated OVA (Fig. 1). The responses of the ISCOMS–OVA-primed group were significantly higher than those of the microparticle-primed group.

Both adjuvants induced significant primary DTH responses and there was no significant difference between the ISCOMS- and PLG-primed groups (Fig. 2).

These results confirm previous findings that a single parenteral immunization of antigen in PLG microparticles or ISCOMS can prime systemic humoral and cell-mediated responses and indicate that they are of comparable magnitude.

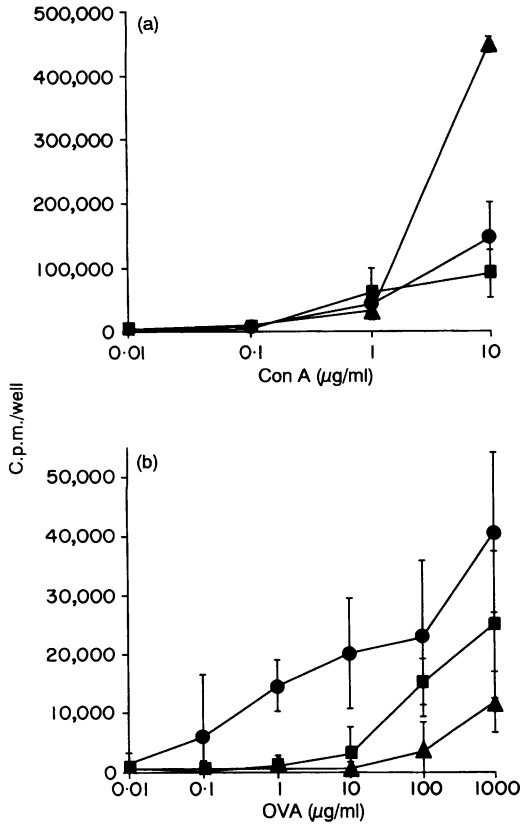

Figure 3. Priming of antigen-specific proliferative T-cell responses by OVA in PLG microparticles or in ISCOMS. Proliferative responses of draining popliteal lymph node cells were assayed 14 days after footpad immunization with either 50 μg OVA in PLG microparticles (■), 5 μg OVA in ISCOMS (●) or saline only (△) after *in vitro* restimulation with (a) Con A or (b) OVA. Results represent mean ± 1 SD c.p.m. values from quadruplicate cultures.

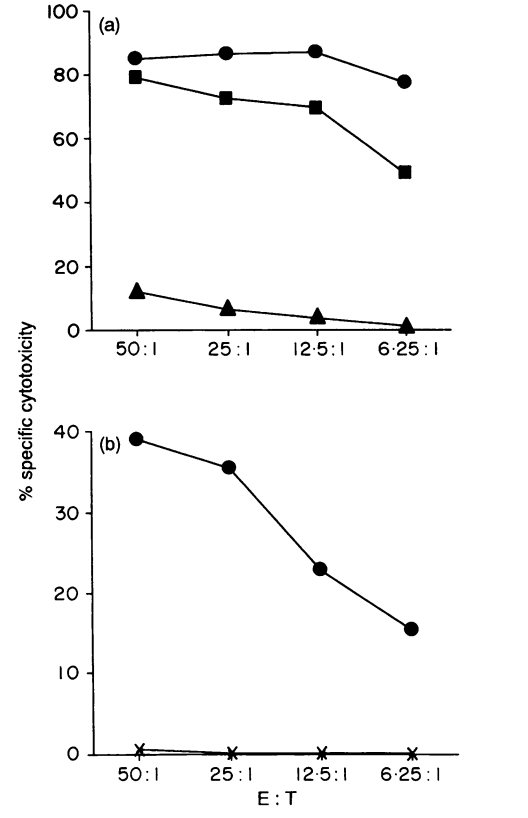

Figure 4. Induction of OVA-specific CTL responses following parenteral immunization with OVA in PLG microparticles or in ISCOMS. (a) OVA-specific splenic CTL responses were assayed 1 week after i.p. immunization with either 5 μg OVA in ISCOMS (●) or 5 μg (▲) or 100 μg (■) OVA in PLG microparticles and subsequent restimulation *in vitro* with EG7.OVA cells. (b) Local OVA-specific CTL responses in draining popliteal lymph node cells measured 4 days after footpad immunization with either 50 μg OVA in PLG microparticles (×) or 5 μg OVA in ISCOMS (●) and subsequent *in vitro* restimulation with rhIL-2.

Priming of antigen-specific proliferative responses by OVA in PLG microparticles or ISCOMS

We next examined the abilities of PLG-entrapped OVA and ISCOMS-OVA to prime for antigen-specific proliferative responses *in vitro*. Popliteal lymph node cells isolated 2 weeks after subcutaneous immunization responded strongly to stimulation with Con A *in vitro*. (Fig. 3a). In addition, lymph node cells from mice primed with either ISCOMS–OVA or OVA in PLG microparticles exhibited marked proliferative responses to restimulation with exogenous soluble OVA (Fig. 3b). However, these responses were only evident at the highest concentrations of OVA with microparticle-primed cells, whereas ISCOMS-primed cells responses at concentrations of as little as 1–10 μg OVA/ml. Unprimed cells showed no response to OVA *in vitro*.

Thus, although both vectors prime local proliferative T-cell responses, ISCOMS appear to be more potent than PLG microparticles for this purpose.

Induction of OVA-specific CTL by parenteral immunization with OVA entrapped in microparticles or in ISCOMS

ISCOMS are powerful vehicles for the induction of antigen-specific CTL when administered either orally or systemically[9,10,15,17] and we next examined whether PLG microparticles were also capable of generating similar CTL responses.

As we have shown previously,[9] spleen cells from mice primed with 5 μg OVA in ISCOMS i.p. 7 days before and restimulated with EG7.OVA cells *in vitro* exhibited very high levels of OVA-specific CTL activity, even at E:T ratios as low as 6·25:1 (Fig. 4a). In contrast, mice immunized with the same amount of OVA in PLG microparticles had no OVA-specific CTL activity in the spleen. Mice immunized with a much larger dose of OVA (100 μg) in PLG microparticles did generate OVA-specific CTL responses, although these were much reduced at lower E:T ratios when compared with those found using ISCOMS–OVA-primed cells. These results suggest that ISCOMS are much more efficient inducers of CTL responses than PLG microparticles.

To test this hypothesis further, we examined directly for the presence of CTL precursors in the draining lymph node following footpad immunization with 100 μg OVA in microparticles or 5 μg OVA in ISCOMS, without further restimulation with antigen. As shown in Fig. 4b, ISCOMS–OVA-primed cells showed substantial OVA-specific CTL activity in this assay,

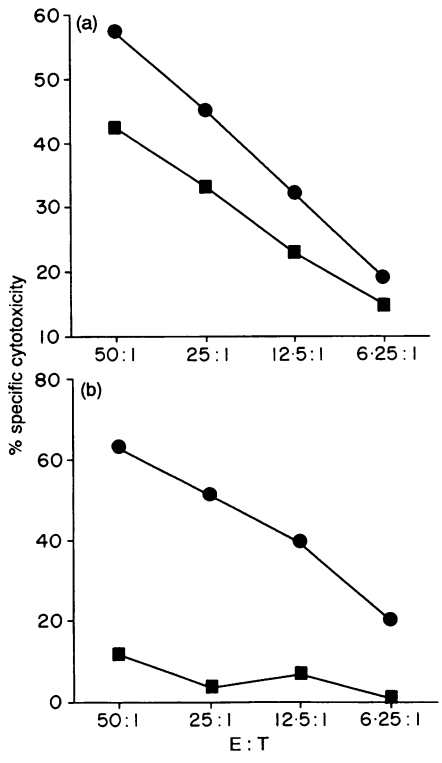

Figure 5. Induction of OVA-specific systemic CTL responses by oral immunization with OVA in PLG microparticles (■) or in ISCOMS (●). Splenic CTL responses were assayed after (a) six feeds or (b) three feeds of either 100 μg OVA in PLG microparticles or 50 μg OVA in ISCOMS.

whereas no CTL activity could be detected in the PLG microparticle-primed group.

These results confirm that the ability of PLG microparticles to prime antigen-specific CTL responses when administered parenterally, is of relatively low efficiency compared with ISCOMS.

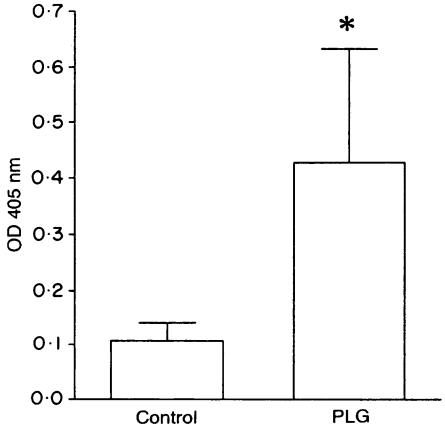

Figure 6. Oral immunization with OVA in PLG microparticles induces OVA-specific intestinal IgA. OVA-specific IgA levels were analysed in intestinal washes by ELISA 1 week after six feeds of either 100 μg OVA in PLG microparticles or saline only. Results shown are means ± 1 SD for six mice per group. $*P < 0.05$ versus control.

Induction of OVA-specific CTL by oral immunization with OVA in PLG microparticles or ISCOMS

We next compared the ability of PLG and ISCOMS to induce systemic CTL responses when administered orally. As we have shown previously,[15] mice fed 100 μg OVA in ISCOMS six times over a 10-day period developed high levels of OVA-specific CTL in the spleen. Similar levels were found in mice fed the same amount of OVA in PLG microparticles (Fig. 5a). However, as we found after systemic immunization, the CTL responses primed by oral administration of ISCOMS–OVA were greater than those observed in mice fed OVA in PLG microparticles. Furthermore, whereas mice fed ISCOMS–OVA on only three occasions still exhibited strong splenic CTL responses, mice receiving three oral immunizations with OVA in PLG microparticles had only very low levels of OVA-specific CTL activity (Fig. 5b).

Thus, PLG microparticles are able to prime potent CTL responses when administered orally, but again, ISCOMS are much more efficient by this route.

Induction of intestinal IgA responses by feeding OVA in PLG microparticles

Previous studies have demonstrated salivary IgA and systemic IgG antibody responses after oral immunization of OVA entrapped in PLG microparticles.[7] Mice fed six times with 100 μg OVA in PLG microparticles also had significant OVA-specific IgA in intestinal secretions (Fig. 6), confirming that PLG microparticles are potent inducers of local and systemic antibody responses when administered orally.

DISCUSSION

These studies show that oral or parenteral immunization with antigen entrapped in PLG microparticles primes a wide range of humoral and cell-mediated immune responses, both locally and systemically. This extends previous reports of the adjuvant activity of PLG microparticles[5-7] and emphasizes their potential as oral vaccine vectors.

The most novel finding of our study was that PLG microparticles primed antigen-specific systemic CTL responses after either parenteral or oral administration. This has never been demonstrated previously using PLG microparticles. Although we have not yet characterized the CTL induced by PLG–OVA, we consider these will be conventional class I major histocompatibility complex (MHC)-restricted CTL. The EG7.OVA cells do not express class II MHC antigens[12] and the responses stimulated by PLG appear very similar to those found after immunization with OVA in ISCOMS, which are known to be due to $CD8^+$ T cells which recognize the octamer motif (OVA 257–264) presented by $H\text{-}2K^b$ class I MHC molecules.[9,17,18] It is also unknown how PLG-entrapped antigen could stimulate class I MHC-restricted CTL. Exogenously administered antigens usually do not enter the endogenous pathway of antigen processing necessary for priming CTL and most of the artificial vectors which overcome this constraint are lipophilic in nature.[19-22] Although this property is consistent with the ability of ISCOMS to stimulate $CD8^+$ T cells,[9,17] it seems unlikely to explain our findings with PLG. However, there is increasing evidence that phagocytosed antigens are

particularly efficient at stimulating class I MHC-restricted T cells[23-25] and it is known that PLG particles are phagocytosed rapidly *in vivo*.[26,27] Investigating the antigen presentation pathways used by PLG would be important not only for elucidating the vaccine potential of PLG microparticles, but could also prove useful for fundamental studies of the endogenous pathway of antigen processing.

In our hands, the CTL responses induced by ISCOMS microparticles were higher than those induced by PLG, requiring fewer or lower doses of antigen. It was also impossible to detect CTL activity in PLG–OVA-immunized mice without *in vitro* restimulation with antigen, whereas the lymph nodes of ISCOMS-immunized animals contained pre-CTL which could be activated by IL-2 alone. This suggests that OVA in ISCOMS primes a higher frequency of antigen-specific CTL. Although the OVA in the ISCOMS differed from that in the PLG by having been palmitified, our previous work has shown that palmitification does not influence the immunogenicity of OVA for class I MHC-restricted T cells.[9,15] Therefore we believe that the additional lipids and saponin present in the ISCOMS allow them to enter cytosolic processing pathways with greater efficiency, perhaps by interacting directly with cellular or endosomal membranes. It is also possible that ISCOMS and PLG microparticles may associate with distinct populations of antigen-presenting cells.

Subcutaneous administration of both OVA in PLG microparticles and in ISCOMS primed for strong OVA-specific IgG and DTH responses after challenge with heat-aggregated OVA. In addition, these mice had excellent *in vitro* OVA-specific proliferative responses in the draining lymph nodes. Together, these findings confirm the similar adjuvant effects of PLG microparticles and ISCOMS and indicate that a single immunization of antigen in either of these vectors results in strong priming of antigen-specific T and B cells.

The second novel finding of our study was that oral immunization with OVA in PLG induced antigen-specific IgA responses in the small intestine itself. This is consistent with previous studies which showed serum IgG and salivary IgA antibody responses in mice fed PLG–OVA.[7,26] These results underline the potential of PLG microparticles for stimulating local and systemic immunity by the oral route.

As we have noted, ISCOMS appeared superior to PLG microparticles in inducing serum IgG responses, CTL responses and T-cell proliferation, despite the fact that a 10-fold lower dose of antigen was used with ISCOMS. For a number of reasons however, we cannot conclude that ISCOMS have a greater potential for use as vaccine vectors. As PLG microparticles have marked delayed release properties, it would be important to determine the kinetics of the primary and memory responses induced by the two vectors. In addition, it will now be important to explore their ability to induce protective immunity using antigens of pathogenic relevance. Finally, economic factors such as ease and cost of manufacture, as well as practical considerations such as stability and possible toxic side-effects must be taken into account when designing a novel vaccine. Nevertheless, our current findings suggest that ISCOMS may provide a more suitable vector for use with antigens which are not in plentiful supply, such as recombinant protein antigens or peptides.

In summary, these results confirm and extend previous findings and suggest that both PLG microparticles and ISCOMS have a great deal of potential as vaccine vectors of the future, particularly in the field of oral vaccination. In addition their potent adjuvant properties should make them useful in helping elucidate the mechanisms involved in immune responses *in vivo*, especially in the induction of CTL responses.

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