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The demonstration of an essential role for macrophages in the *in vivo* generation of IgG2a antibodies

J. M. BREWER, J. RICHMOND* & J. ALEXANDER *Department of Immunology, University of Strathclyde, and *Department of Pathology, The Royal Infirmary, Glasgow, UK*

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SUMMARY

BALB/c mice were depleted of macrophages by intravenous inoculation of dichloromethylene diphosphonate entrapped in liposomes 24 h before primary and 24 h before secondary sensitization intravenously with 100 μg bovine serum albumin (BSA) or ovalbumin (OVA). The effectiveness of macrophage depletion was confirmed by immunocytochemistry. Five days and 14 days after secondary challenge with BSA, plasma samples from these and control mice inoculated with empty liposomes were examined for the production of BSA-specific IgG1 and IgG2a antibodies. Macrophage depletion resulted in a significantly increased production of the Th2 lymphocyte-associated IgG1 isotype, while the production of specific IgG2a antibodies, produced under the influence of Th1 cells, was totally ablated. Similar results were obtained when OVA was used as the test antigen. Furthermore, analysis of interferon-gamma (IFN-γ) production after antigen or concanavalin A (Con A) restimulation *in vitro* indicated that macrophage depletion *in vivo* significantly reduced production of this Th1 cell-associated cytokine. These results provide strong *in vivo* and *in vitro* evidence for the macrophage being the antigen-presenting cell population responsible for Th1 cell activation.

Keywords macrophage Cl2MDP IgG2a Th1 interferon-gamma

INTRODUCTION

The differential capacity of distinct antigen-presenting cell (APC) populations to present antigen to and costimulate different $CD4^+$T cell subsets has been extensively studied *in vitro*. While the studies of Gajewski *et al.* [1] and Abbas *et al.* [2] suggest that macrophages may preferentially stimulate the expansion of Th1 cells, and B cells preferentially stimulate Th2 cells, contradictory results have been reported from other *in vitro* studies [3–9]. However, these studies fail to take into account the microenvironment of the lymphoid tissues, which undoubtedly play a critical role in determining the outcome or polarization of an immune response [10,11]. This can only be determined by investigations *in vivo*.

Selective depletion of APC populations may provide a more relevant indication of the role played by the different cell populations. Macrophage depletion *in vivo* can be performed by the introduction of a variety of agents such as silica [12,13], carageenan [14], and asbestos [12]. However, these substances are not ideal, as they also have a variety of effects on lymphoid cells as well as macrophages, and their effects on macrophages are extremely variable [11]. The *in vivo* macrophage elimination technique described by van Rooijen [15] employs a liposome encapsulated drug, dichloromethylene diphosphonate (clodronate; C12MDP). When liposomes containing C12MDP are ingested by macrophages, lysosomal lipases break down the liposomes releasing C12MDP [11,15,16]. The released drug then acts on the phagocytic cell resulting in cell death, possibly due to the calcium chelating activity of C12MDP [11]. In the event of any leakage from liposomes or dying cells, free C12MDP is rapidly eliminated by the kidney *in vivo*, thus limiting the effect of the drug on surrounding cell populations [11]. Elimination of macrophages in the spleen and liver 24 h after i.v. administration of liposomal C12MDP has been confirmed by histological examination [11,15], and the kinetics of macrophage repopulation of depleted organs also studied [17]. While repopulation of the murine spleen by red pulp macrophages can be observed approximately 1 week after administration of liposomal C12MDP, repopulation by marginal metallophilic macrophages and marginal zone macrophages takes 2 weeks and 1 month, respectively [17].

Although the effects of macrophage elimination by liposomal C12MDP on immune responses to liposome-associated antigens have been extensively studied ([18,19], reviewed [11,16]), the influence of macrophage elimination by this or

Correspondence: Dr J. Brewer, Department of Immunology, University of Strathclyde, The Todd Centre, 31 Taylor Street, Glasgow G4 0NR, UK.

other techniques on Th1/Th2 cell activation has not as yet been examined. In this study we therefore monitored the effects of liposomal C12MDP-mediated elimination of macrophages on IgG1 and IgG2a production, associated with Th2 cell and Th1 cell activation, respectively [20,21], to the standard antigens bovine serum albumin (BSA) and ovalbumin (OVA). We also examined the production of the Th1 cell-associated cytokine interferon-gamma (IFN-γ) [22], after antigen-specific or concanavalin A (Con A) restimulation *in vitro* following *in vivo* immunization with OVA or purified protein derivative of tuberculin (PPD) and macrophage depletion.

MATERIALS AND METHODS

Preparation of liposomal C12MDP

Dichloromethylene diphosphonate (clodronic-acid disodium salt tetrahydrate; C12MDP) was a gift from Boehringer Mannheim (Mannheim, Germany). Preparation of liposomes containing C12MDP has been described previously [15]. Egg phosphatidyl choline (75 mg; Sigma, Poole, UK) and 11 mg of cholesterol (Sigma) were dissolved in approximately 10 ml of chloroform in a 500-ml round-bottomed flask. The chloroform was then removed by low vacuum rotary film evaporation at 37°C to leave a thin film on the sides of the flask. The film was subsequently dispersed with 10 ml of PBS pH 7·4 or PBS containing 2·5 mg of C12MDP by gently swirling for 15 min at room temperature. After complete removal of the lipid film from the sides of the flask, the resulting suspension was incubated for 2 h at room temperature. After this period, the suspension was placed in a sonicating waterbath (50 Hz; Mettler Electronics, Pasadena, CA) and sonicated at room temperature for 3 min. After a further 2-h incubation at room temperature, free C12MDP was removed by centrifuging the suspension at 100 000 *g* for 30 min at 16°C. The resulting pellet was washed in PBS as above and then resuspended in 4 ml of PBS.

Animals and inoculations

Female BALB/c mice (four to six per group) of between 6 and 8 weeks and bred at the University of Strathclyde were used. Each mouse was injected intravenously with 0·2 ml of either empty liposomes or liposome containing C12MDP. Twenty-four hours later, each animal was inoculated intravenously with 100 μg of BSA or OVA (Sigma) in 0·1 ml PBS. This procedure was repeated 27 days after the first liposome inoculation. Blood samples were collected on days 5 and 14 after secondary inoculation with BSA and 14 days after secondary inoculation with OVA.

Immunocytochemistry

The presence or absence of macrophages was determined by immunocytochemical analysis of spleens removed from mice 5 days after i.v. administration of liposomes containing C12MDP or PBS. Cryostat sections (5 μm) of spleen were air dried onto slides coated with 3-aminopropyltriethoxysilane (Sigma). These preparations were then fixed in acetone, dried and then washed in 50 mM Tris-buffered saline (TBS; pH 7·6). Subsequently, spleen sections were incubated for 2 h at room temperature with either a 1 : 10 dilution (in TBS) of biotinylated MoAb specific for macrophage antigen F4/80 ([23]; Serotech, Oxford, UK) or a 1 : 10 dilution of SER-4 MoAb specific for the macrophage sheep erythrocyte receptor [24]. After washing in TBS for 10 min, spleen sections were incubated for 2 h at room temperature with either horseradish peroxidase (HRP)/streptavidin conjugate for anti-F4/80 (1 : 300 dilution in TBS; Boehringer Mannheim, Lewes, UK) or HRP/rabbit anti-rat conjugate (Vector Labs, Peterborough, UK) for SER-4. Sections were washed as above and peroxidase activity was revealed by a 10-min incubation at room temperature with 3,3′-diaminobenzidine (DAB; Sigma), which was followed by washing under running tap water. Sections were weakly counterstained with haematoxylin and dehydrated through alcohol and xylene before mounting in DePeX (BDH, Glasgow, UK).

ELISA

Enzyme-linked immunoassays were performed as described by Brewer & Alexander [25] to detect specific IgG1 and IgG2a in plasma diluted 1 : 1000 and 1 : 100, respectively. Flat-bottomed polystyrene plates (Dynatech, Alexandria, VA) were coated with 100 μl BSA (1 μg/ml in 0·02 M Tris/HCl pH 9·0) per well and incubated overnight at 4°C. Wells were then washed three times with Tris/Tween buffer (0·02 M Tris, 0·05% v/v Tween 20; pH 7·4), then 150 μl of BLOTTO (5% non-fat dried milk prepared in Tris/Tween buffer; [26]) was added to each well and incubated for 1 h at 37°C. Wells were washed as above and 100-μl samples of plasma diluted in PBS/Tween (0·05% v/v Tween 20 pH 7·4) were added to duplicate wells. Samples were incubated for 1 h at 37°C, then the wells were washed as before. Aliquots of 100 μl of HRP-conjugated goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL) diluted 1 : 8000 or 1 : 800 respectively in 75% PBS : 25% sheep serum (v/v) were added to each well. The wells were incubated for 1 h at 37°C before washing as above. Substrate solution was prepared by addition of 5 μl of hydrogen peroxide and 250 μl of 6 mg/ml tetramethylbenzidine in dimethylsulphoxide to 25 ml of 0·1 M sodium acetate solution pH 5·5. Each well had 100 μl of substrate solution added before incubation for 15 min in darkness at room temperature. The reaction was stopped by addition of 10% sulphuric acid (v/v) and the absorbance at 450 nm was measured on a Titertek Multiskan (Flow Labs, Irvine, UK). Results were expressed as mean absorbance detected at 450 nm ± s.e.m. OVA-specific IgG2a was detected by a similar protocol, although 64 μg/ml OVA was used to coat plates, and serial dilutions of plasma were prepared so that an endpoint dilution could be determined. Comparisons between groups were performed using a Mann–Whitney *U*-test.

IFN-γ assay

BALB/c mice were inoculated intravenously with liposomal C12MDP or liposomal PBS followed 24 h later by 100 μg OVA or 10 μg PPD (Central Veterinary Laboratory, Weybridge, UK) in PBS as described above. One week after treatment, mouse spleens were aseptically removed and placed in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0·05 mM β-mercaptoethanol (GIBCO, Paisley, UK). Cell suspensions were prepared by gently teasing the spleens apart with forceps, after which the cell suspensions were centrifuged at 200 *g* for 5 min. Erythrocytes were removed by resuspending the pellet in 3 ml Boyles Solution (0·17 M Tris, 0·16 M ammonium chloride; BDH) and incubating for 3 min at 37°C. After two washes in RPMI

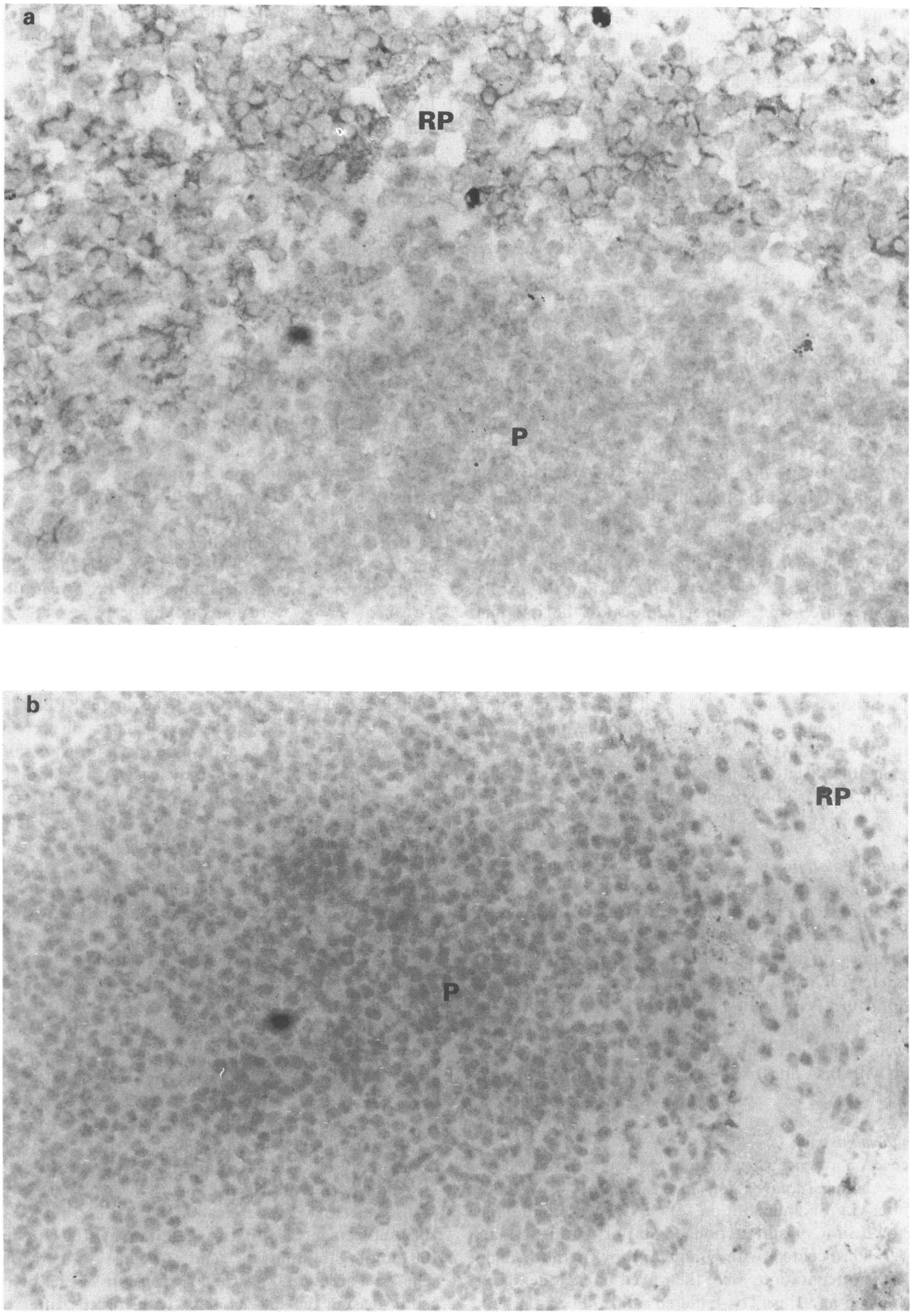

Fig. 1. Sections of mouse spleen stained by immunoperoxidase for the expression of macrophage-specific antigen F4/80 and weakly counterstained with haematoxylin to reveal the morphology of the spleen. F4/80-positive macrophages can be detected in the red pulp (RP) but not in the periarteriolar lymphoid sheath (P) of spleens removed from mice treated with liposomes containing PBS (a). F4/80-positive macrophages were not evident in similar regions of the spleen removed from mice treated with liposomal clodronate (b) (×500).

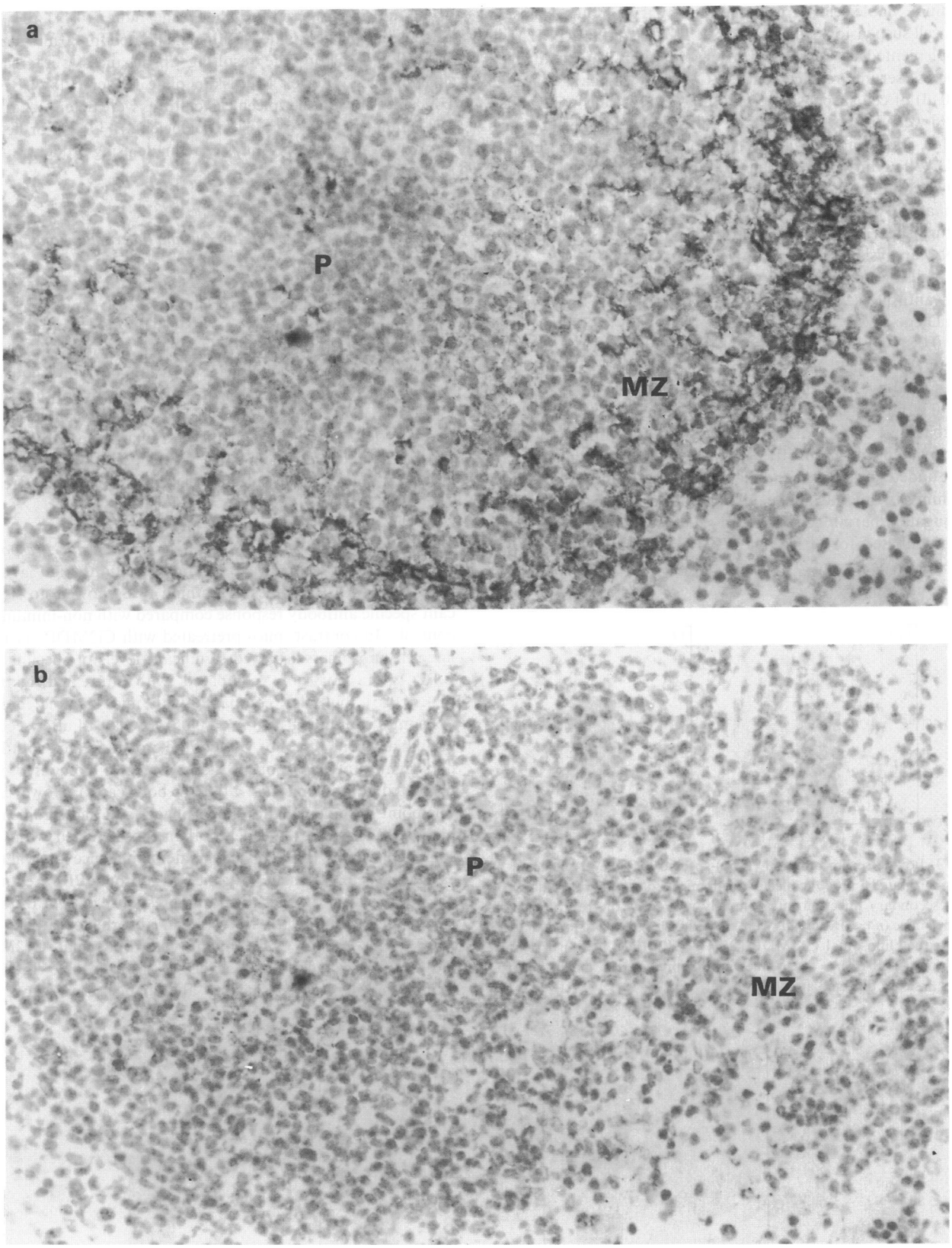

Fig. 2. Sections of mouse spleen stained by immunoperoxidase for the expression of macrophage SER-4 antigen and weakly counterstained with haematoxylin to reveal the morphology of the spleen. SER-4 antigen-positive macrophages could be detected in the marginal zone (MZ) surrounding the periarteriolar lymphoid sheath (P) of spleens removed from mice treated with liposomes containing PBS (a). SER-4 antigen-positive macrophages were not evident in similar regions of the spleen removed from mice treated with liposomal clodronate (b). (×500).

medium, viable cells were enumerated by trypan blue exclusion and cell suspensions adjusted to 5×10^6 cells/ml in RPMI 1640 supplemented as above and also containing 10% controlled process serum replacement-2 (CSPR-2; Sigma). Aliquots (100 μl) of cell suspension containing 5×10^5 cells were added to 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA) and 100 μl/well aliquots of Con A (5 μg/ml) or PPD (30 μg/ml) were added in triplicate. Control wells had 100 μl complete RPMI added. Cells were then incubated for 60 h at 37°C, 5% CO_2, after which 150-μl aliquots of cell culture supernatants were removed and stored at −70°C for IFN-γ analysis.

Wells of a microtitre plate were precoated overnight at 4°C with anti-mouse IFN-γ MoAb (50 μl, 2 mg/ml; Pharmingen, San Diego, CA), then washed three times with PBS/Tween (pH 7·4, 0·05% Tween 20) and blocked with 200 μl 10% fetal calf serum (FCS) in PBS for 60 min at 37°C. After washing three times as above, 150-μl samples of supernatants and standards (0–70 U/ml IFN-γ) were added in duplicate to wells and incubated at 37°C for 2 h. After washing four times, 100 μl of biotinylated anti-mouse IFN-γ MoAb (1 μg/ml; Pharmingen) were added to each well and incubated for 45 min at 37°C. After washing six times as above, alkaline phosphatase–streptavidin conjugate (Pharmingen) diluted 1 : 2000 was added to each well and incubated for 30 min at 37°C. Wells were washed eight times as above and 100 μl of paranitrophenyl-phosphatase (1 mg/ml; Sigma) prepared in glycine buffer (0·1 M pH 10·4) added per well. The plate was then incubated for 30 min at 37°C in darkness before the resulting absorbances were read at 405 nm on a Titertek Multiskan plate reader (Flow). IFN-γ concentrations in the spleen cell cultures were determined from the standard curve (regression coefficient, $r = 0{\cdot}990$ or better). Comparisons between groups were made using Student's t-test.

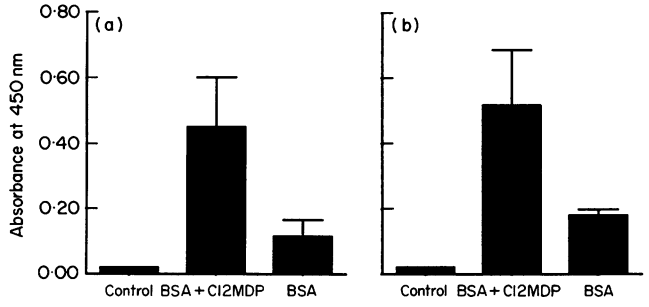

Fig. 3. Bovine serum albumin (BSA)-specific IgG1 levels detected in plasma (diluted 1 : 1000) collected from mice 5 days (a) and 14 days (b) after secondary injection intravenously with BSA or from untreated control mice ($n = 5$). Animals were treated 24 h before BSA inoculation with either liposomal C12MDP (BSA + C12MDP; $n = 6$) or empty liposomes (BSA; $n = 4$). Values are expressed as mean absorbances ± s.e.m. detected at 450 nm.

RESULTS

Liposomal C12MDP pretreatment and macrophage populations in the spleen

Immunocytochemistry of spleen sections indicated that following treatment with liposomes containing PBS, expression of the macrophage-specific antigen, F4/80 [23] could be detected in the red pulp (RP) as has been described previously ([27]; Fig. 1a). In contrast, no obvious expression of F4/80 could be detected in spleens removed from mice treated with liposomal Cl2MDP (Fig. 1b). Similarly, SER-4 antigen-positive macrophages could be detected in the marginal zone (MZ) [24,27] of spleens removed from mice treated with PBS/liposomes (Fig. 2a), but not in spleens of Cl2MDP/liposome-treated mice (Fig. 2b).

Liposomal C12MDP pretreatment and secondary IgG1 responses

Five days after secondary BSA immunization (Fig. 3a) mice pretreated with liposomes containing PBS showed no significant specific antibody response compared with non-immunized controls. In contrast, mice pretreated with Cl2MDP encapsulated in liposomes displayed significant levels of specific anti-BSA IgG1, not only compared with untreated controls ($P < 0{\cdot}025$), but also compared with mice pretreated with liposomes containing PBS ($P < 0{\cdot}05$). By day 14 after secondary inoculations (Fig. 3b), both of the groups treated with BSA had significant levels of BSA-specific IgG1 compared with untreated control mice ($P < 0{\cdot}01$ in both cases). However, at this point mice depleted of macrophages still had significantly higher BSA-specific IgG1 levels than those treated with liposomes containing PBS, and at a greater confidence interval than earlier in the experiment ($P < 0{\cdot}01$). Liposome/PBS treatment did not significantly alter the normal response of BALB/c mice to i.v. inoculation with BSA (mean absorbance at day 14, $A_{450} = 0{\cdot}106 \pm 0{\cdot}023$). Treatment with liposomal C12MDP did not alter background levels of BSA-specific IgG1 in unimmunized mice (mean absorbance at day 14,

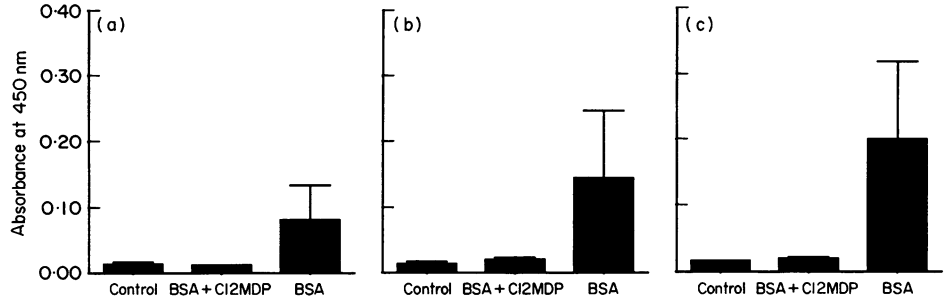

Fig. 4. Bovine serum albumin (BSA)-specific IgG2a levels detected in plasma (diluted 1 : 100) collected from mice 5 days (a), 11 days (b) and 14 days (c) after secondary injection intravenously with BSA or from untreated control mice ($n = 5$). Animals were treated 24 h before BSA inoculation with either liposomal C12MDP (BSA + C12MDP; $n = 6$) or empty liposomes (BSA; $n = 4$). Values are expressed as mean absorbances ± s.e.m. detected at 450 nm.

$A_{450} = 0{\cdot}021 \pm 0{\cdot}001$) compared with unimmunized control mice (mean absorbance at day 14, $A_{450} = 0{\cdot}018 \pm 0{\cdot}002$).

Liposomal C12MDP pretreatment and secondary IgG2a responses
Five days after secondary inoculation (Fig. 4a), mice pretreated with liposomal Cl2MDP did not display significant levels of BSA-specific IgG2a compared with non-immunized controls. In contrast, those mice treated with liposomes containing PBS displayed significant though variable BSA-specific IgG2a ($P < 0{\cdot}05$), which was at a significantly higher level than the mice treated with liposomal Cl2MDP ($P < 0{\cdot}025$). By days 11 (Fig. 4b) and 14 (Fig. 4c) after secondary inoculations, specific IgG2a antibodies were still not detectable in macrophage-depleted mice. Furthermore, those mice pretreated with PBS encapsulated in liposomes had BSA-specific IgG2a levels which were significantly higher than either non-immunized mice (day 11, $P < 0{\cdot}05$; day 14, $P < 0{\cdot}01$) or mice pretreated with liposomal Cl2MDP ($P < 0{\cdot}01$) at day 14. Liposomal/PBS treatment did not significantly alter the normal response of BALB/c mice to i.v. inoculation with BSA (mean absorbance at day 14, $A_{450} = 0{\cdot}196 \pm 0{\cdot}085$). Treatment with liposomal C12MDP did not alter background levels of BSA-specific IgG2a in unimmunized mice (mean absorbance at day 14, $A_{450} = 0{\cdot}015 \pm 0{\cdot}003$) compared with unimmunized control mice (mean absorbance at day 14, $A_{450} = 0{\cdot}016 \pm 0{\cdot}001$).

The inability of macrophage-depleted BALB/c mice to mount specific IgG2a responses was not antigen-dependent, as identical results were obtained using OVA as the test antigen (Fig. 5). Again treatment with liposomal C12MDP did not alter background levels of OVA-specific IgG2a in unimmunized mice (mean reciprocal endpoint 50 ± 0) compared with unimmunized control mice (mean reciprocal endpoint $50 \pm 22{\cdot}4$).

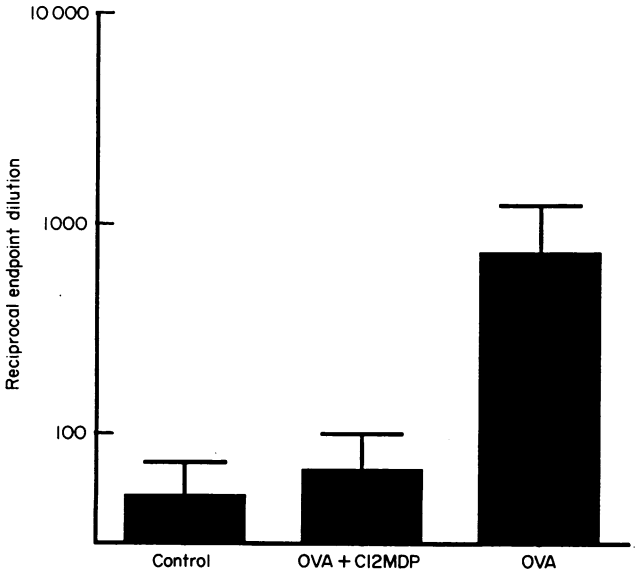

Fig. 5. Ovalbumin (OVA)-specific IgG2a titres detected in plasma collected from mice 14 days after secondary injection intravenously with OVA or from untreated control mice ($n = 5$). Animals were treated 24 h before OVA inoculation with either liposomal C12MDP (OVA + C12MDP; $n = 6$) or empty liposomes (OVA; $n = 6$). Values are expressed as mean reciprocal antibody titres ± s.e.m. as detected by endpoint analysis.

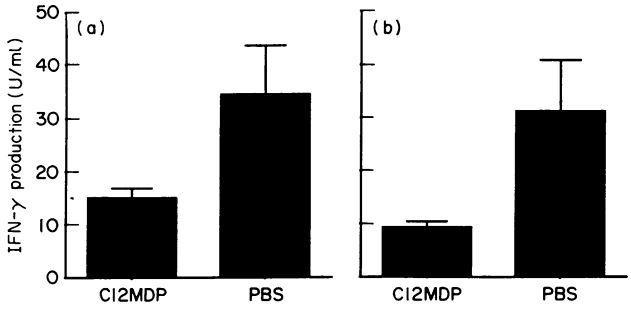

Fig. 6. *In vitro* IFN-γ production by concanavalin A (Con A) (a) or purified protein derivative (PPD) (b) restimulated spleen cell cultures. Mice were treated with either liposomal C12MDP (C12MDP; $n = 5$) or PBS liposomes (PBS; $n = 5$) 24 h before i.v. inoculation of ovalbumin (OVA) or PPD. Spleens were removed 7–9 days later and harvested splenocytes restimulated for 72 h with Con A or PPD before IFN-γ concentration was determined in culture supernatants by ELISA. Values are expressed as mean IFN-γ concentration ± s.e.m.

Liposomal C12MDP pretreatment and in vitro *IFN-γ production*
After Con A restimulation *in vitro*, significantly lower levels of IFN-γ were detected in spleen cell cultures prepared from mice immunized with OVA and pretreated with liposomal C12MDP compared with cultures from similarly immunized mice pretreated with PBS/liposomes (Fig. 6a; $P < 0{\cdot}01$). Similarly, using antigen-specific restimulation in PPD-immunized mice, lower levels of IFN-γ could be detected in spleen cell cultures prepared from mice pretreated with liposomal C12MDP compared with mice pretreated with liposomal PBS (Fig. 6b; $P < 0{\cdot}025$).

DISCUSSION

The results presented in this study indicate that secondary IgG1 responses to intravenously administered BSA are significantly enhanced by i.v. treatment with Cl2MDP encapsulated within liposomes. This observation is consistent with overall results previously obtained using this technique, which indicate that removal of macrophages by treatment with liposomal Cl2MDP enhances secondary IgG responses to another soluble antigen, TNP-KLH [11,16]. Van Rooijen [11,16] proposed that macrophages may actually inhibit the humoral response to soluble antigen, and that competition may exist between macrophages and other APC for antigen. However, the results presented in this study also indicated that IgG2a subclass responses to BSA and OVA are eliminated by Cl2MDP/liposome macrophage depletion. Thus, macrophage-mediated suppression of the humoral immune response may actually be isotype-specific.

The above observation can be explained in terms of selective Th1 or Th2 cell activation by distinct APC populations. If macrophages are the more important cell population in Th1 cell expansion, as suggested by the *in vitro* experiments described herein and by others [1,2,28,29] then *in vivo* elimination of this population by Cl2MDP/liposomes would reduce the antibody subclass associated with Th1-secreted cytokines, namely IgG2a [20,21], as we have in fact observed. Increased IgG1 production observed after macrophage elimination may come about for one or both of the following reasons. First, removal of macrophages may simply increase the quantity of

the antigen reaching other APC populations, such as B cells which have been associated with Th2 cell expansion [1]. This would directly enhance production of Th2 cell-associated cytokines, and subsequently increase IgG1 production [21]. Second, if depletion of macrophages does indeed reduce Th1 cell expansion, as decreased IFN-γ production *in vitro* and IgG2a production *in vivo* would appear to indicate, then this will remove the inhibitory effects of Th1 cell-derived cytokines, most notably IFN-γ, on Th2 cell expansion [22,30,31], resulting ultimately in an increase in IgG1 production.

Despite the large volume of *in vitro* data concerned with the roles of APC populations in the induction of Th1/Th2 cell responses, there is little information available on this phenomenon *in vivo*. Studies employing B cell-deficient (μ-treated) mice have produced conflicting results. The studies of Ron & Sprent [32] and Kurt-Jones *et al.* [33] suggest that following *in vivo* priming, B cells are essential for *in vitro* T cell proliferation. In contrast, the studies performed by Sacks *et al.* [34] suggest that in the BALB/c, but not the C3H/HeJ mouse strain, B cells are responsible for suppressed DTH responses. The present results would appear to be consistent with those described in the BALB/c mouse by Sacks *et al.* [34]. Thus BALB/c mice depleted of macrophages have decreased production of the antibody subclass associated with Th1 cell activity. Conversely, BALB/c mice lacking B cells display increased DTH responses [34], consistent with preferential Th1 cell activation and IFN-γ production. Further evidence that macrophages are the major APC associated with Th1 cell activation is indicated by work using Freund's complete adjuvant (FCA). This adjuvant has previously been shown to preferentially activate Th1 cells [35], and histological analysis of lymph nodes following immunization with FCA has identified IL-2-producing Th1 cells around F4/80-positive macrophages, but not B cells or dendritic cells [36,37].

It is the general consensus of opinion that Th1 cells promote IgG2a production by the generation of IFN-γ [21,38]. However, this lymphocyte subpopulation is not the only source of this cytokine: $CD8^+$ cytotoxic T cells have also been shown to release IFN-γ as well as a Th1 cell pattern of cytokines in general [39]. Although both macrophages [40] and dendritic cells [41] have been proposed as accessory cells in the generation of $CD8^+$ T cells, a large number of recent studies implicate macrophages as the most important cell population in processing foreign antigen for class I MHC presentation [42–46]. Significantly, *in vivo* depletion of macrophages has been shown to ablate $CD8^+$ cytotoxic T cell responses induced by intravesicular antigen [45].

Thus our results indicate that the macrophage in its role as an APC plays a crucial role in the production of IgG2a antibodies *in vivo* and antigen-specific and non-specific IFN-γ production *in vitro*. While *in vivo* this response is most probably influenced ultimately by IFN-γ produced by an expanding Th1 cell population, a role for $CD8^+$ cytotoxic T cells cannot be discounted.

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