The mode of protein antigen administration determines preferential presentation of peptide–class II complexes by lymph node dendritic or B cells

Jean-Charles Guéry 1, Francesco Ria 2, Francesca Galbiati, Simona Smiroldo and Luciano Adorini

Roche Milano Ricerche, Via Olgettina, 58, 20132 Milano, Italy

1Present address: INSERM U28, Hôpital Purpan, 31059 Toulouse, France
2Present address: Istituto di Patologia Generale, Università Cattolica del Sacro Cuore, 00168 Roma, Italy

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Abstract

We have compared the capacity of dendritic cells (DC) and B cells to present peptide–class II complexes following administration of protein in adjuvant or in soluble form. Three different antigen-presenting cell (APC) populations were separated from draining lymph node cells from mice immunized s.c. with hen egg-white lysozyme (HEL) in adjuvant or with adjuvant only followed by soluble HEL: DC (N418 F, class II F, B220 –, low buoyant density), large B cells (B220 –, low buoyant density) and small B cells (B220 –, high buoyant density). HEL peptide–class II complexes displayed by these APC were evaluated by their capacity to activate HEL-specific T hybridoma cells. Following immunization with HEL in adjuvant, DC are the only lymph node APC population expressing detectable HEL peptide–class II complexes. Conversely, after i.v. administration of soluble HEL in mice previously injected with adjuvant only, lymph node B cells are much more efficient than DC in presenting peptide–class II complexes to T cells. Therefore, different modes of protein antigen administration lead to selective expression of antigenic complexes by different APC populations. These data correlate with the observation that, unlike B cells, DC recruited in lymph nodes of mice injected with adjuvant only present in vitro processed protein antigen much less efficiently than synthetic peptides, probably as a consequence of their maturation in vivo.

Introduction

The activation of CD4+ T cells is initiated by the recognition of peptide–class II MHC complexes on the surface of antigen-presenting cells (APC) like macrophages, dendritic cells (DC) and B cells (1,2). Among the different APC populations capable of presenting peptide–class II MHC complexes, DC and B cells have been studied extensively, but their relative role in the presentation of protein antigen and CD4+ T cell priming in vivo is still controversial. Using mice lacking B cells, presentation to CD4+ T cells of antigenic complexes derived from processing of protein antigen administered in adjuvant was found in some studies to require B cells (3), whereas in others B cells were found not critical and antigenic complexes were presumably presented by DC (4). Similarly, administration of soluble protein to normal mice was found to lead to selective expression of antigenic complexes either by antigen-specific B cells (3) or by DC (5).

DC represent a system of highly specialized APC present, in different stages of maturation, in the circulation as well as in lymphoid and non-lymphoid organs (6,7). Immature DC, such as Langerhans cells in the skin, are found in non-lymphoid tissues, where they exert a sentinel function. After antigen uptake, they migrate through the afferent lymph to T-dependent areas of lymphoid organs where priming of naive T cells may occur (6,8). Based on in vitro data, it has been hypothesized that during this process they mature into potent APC by increasing their immunostimulatory properties while losing their antigen capturing capacity (9,10). Thus, mature DC should have in vivo an impaired capacity to endocytose...
protein antigens but will present very efficiently peptides derived from proteins which have been previously endo-
cytosed at the site of inflammation.

To address this point, we have compared the relative capacity of DC and B cells recruited in lymph nodes during the inflammatory response induced by adjuvant administration to present protein antigen administered in different forms. We have previously shown that immune lymph node cells (LNC) from mice immunized with hen egg-white lysozyme (HEL) in adjuvant display HEL peptide–MHC class II complexes able to stimulate, in the absence of any further antigen addition, specific T hybridoma cells (11,12).

In the present paper, HEL-specific T hybridoma cells were used to read-out expression by DC and B cells of antigenic complexes derived from processing of native HEL, either given s.c. in adjuvant or in soluble form i.v. Phenotypic analysis of the stimulatory APC in immune lymph nodes confirms our previous study demonstrating that following s.c. administration of HEL in adjuvant DC are the only APC expressing detectable HEL peptide–class II complexes (13). Conversely, when HEL is administered in soluble form i.v. to mice previously injected with adjuvant only, lymph node B cells are much more efficient than DC in the presentation of HEL peptides. These results demonstrate that protein antigen injected in soluble form is presented best by B cells, whereas the same protein is presented only by lymph node DC when administered in adjuvant. Therefore, different protocols of protein antigen administration lead to expression of peptide–class II complexes by different APC. These results also suggest maturation of DC in vivo.

Methods

Mice, antigens and immunizations
Female BALB/c mice (2–3 months old; Charles River, Calco, Italy) were used. HEL, reconstituted three times, and ovalbumin (ova) grade were obtained from Sigma (St Louis, MO). HEL peptide sequence 105–120 (MN-WWAWRNRCGKTDV) was purchased from NeoSystem (Strasbourg, France). Mice were immunized s.c. into the hind footpads with the indicated amount of HEL dissolved in PBS and emulsified vol/vol in incomplete Freund’s adjuvant (IFA) (Difco, Detriot, MI). In addition, mice were injected with 1 nmole OVA-IFA in to the hind footpads and 5 days later received 100 nmols soluble HEL i.v. 3 h before sacrifice. Enrichment of APC populations
Immune LNC were depleted of T cells by cytotoxic elimination with HO-13-4 anti-Thy-1.2 mAb (TIB 99) followed by rabbit complement (Low-Tox M; Cedarlane, London, Canada). Low and high buoyant density APC were prepared from T cell-depleted LNC by centrifugation over a discontinuous Percoll (Pharmacia LKB, Uppsala, Sweden) gradient containing 55–60% and 70% layers. Cells at the medium/55–60% and 60/ 70% interface were collected separately and referred to as low- and high-density APC respectively. B cells in the low-density population were further depleted by magnetic cell sorting using the MiniMACS magnetic separation system following the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were incubated with B220-coated microbeads, and then separated into B220+ and B220− fractions on MiniMACS separation columns.

Flow cytometry
Cells were double stained by incubating them with optimal concentrations of FITC–14.4.4S (anti-I-E) mAb, biotin-conjugated N418 (anti-CD11c) or 6B2 (anti-B220) mAb for 30 min at 4°C in PBS containing 5% FCS, 0.1% sodium azide and 1% normal rat serum to inhibit binding to FcR. 14.4.4S and 6B2 mAb were purchased from Pharmingen (San Diego, CA). N418 is an hamster mAb specific for mouse splenic DC recognizing the p150/90 leukocyte integrin, likely the mouse CD11c molecule (14,15), and was obtained from the ATCC (HB-224; Rockville, MD). Biotinylated mAb were revealed using phycoerythrin–streptavidin (Southern Biotechnology Associates, Birmingham, AL). Analysis was performed on a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Data were collected on 5000–10,000 viable cells as determined by forward light scatter intensity and propidium iodide exclusion, and analyzed using Lysys II software.

Assay for antigen-presenting activity
The antigen-presenting activity of lymph node APC was assessed as previously described (11) using the T cell hybridomas 1H11.3 (I-Eβ, HEL108–116) (16) and 2G12.1 (I-Ad, B2M26–39) (17). Briefly, mice were immunized into the hind footpads with the indicated amount of HEL emulsified in IFA. Three to ten days after immunization, the draining popliteal lymph nodes were removed and APC enriched as detailed above. In addition, mice were injected with 1 nmole OVA-IFA into the hind footpads and 5 days later received 100 nmols soluble HEL i.v. 3 h before sacrifice. Lymph node DC, large and small B cells were cultured in duplicate or triplicate at the indicated cell doses with appropriate T cell hybridomas (5×104 cells/well) in 96-well culture plates (Costar). Culture medium was RPMI 1640 (Gibco, Basel, Switzerland) supplemented with 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 50 µg/ml gentamicin (Sigma) and 10% FCS (Gibco). After 24 h of culture, 50 µl aliquots of supernatants were transferred to microculture wells containing 104 CTL1 cells and, after an additional 24 h incubation, the presence of T cell growth factors, mainly IL-2, was assessed by [3H]thymidine incorporation during the last 5 h of culture. In some experiments, IL-2 concentration was determined using a two-site sandwich ELISA with paired mAb (JES6–1A12, JES6–5H4) purchased from Pharmingen as described elsewhere (13). IL-2 was quantified from two to three titration points using standard curves generated by purified recombinant mouse IL-2 and results expressed as cytokine concentration in pg/ml. The detection limit of the assay was 10 pg/ml.

Results

Enrichment of DC and B cells
By a combination of Percoll gradient centrifugation and magnetic cell sorting, we separated lymph node APC into three populations: small B cells, large B cells and DC-enriched
cells (Fig. 1). These populations were characterized by their expression of MHC class II, B220 and N418 molecules. The high-density population contains >95% B220⁺, class II⁺ N418⁺ cells and is referred to as small B cells. Large B cells, obtained from the low-density population by positive selection using B220 mAb-conjugated magnetic particles, are >95% B220⁺, class II⁺, N418⁺. The negatively selected fraction, depleted of B220⁺ cells, is enriched in class II⁺, N418⁺ cells (50–70%) and is referred to as DC-enriched population.

The mode of protein antigen administration determines preferential presentation of peptide–class II complexes by DC or B cells

We have recently shown that following administration of HEL in adjuvant antigenic complexes are only detectable on lymph node DC, whereas B cells are devoid of antigen-presenting activity (13). In the present paper, we demonstrate that DC isolated 3 and 6 days after injection of HEL in adjuvant present very efficiently the HEL epitope 108–116 bound to I-E^d molecules whereas its presentation decreases considerably by day 10 after priming. At all time points tested B cells, either large or small, fail to present or present very inefficiently HEL108–116/I-E^d complexes to T hybridoma cells (Fig. 2). To determine whether the mode of protein antigen administration determines preferential presentation of peptide–class II complexes by DC or B cells, we have analysed the capacity of resident lymph node APC to present antigenic complexes following i.v. administration of HEL in mice previously injected with adjuvant. Injection of adjuvant recruits APC in the draining lymph nodes. Five days after administration of soluble HEL in the hind footpads popliteal lymph nodes harvested from nine mice yielded a total of 35 × 10⁶ cells which, after T cell depletion, were reduced to 1.6 × 10⁶. Due to the very low number of cells recovered we could not proceed further with cell enrichment. Cytofluorimetric analysis revealed that this cell population contained 4% N418⁺ cells, which represents a yield of 7 × 10⁵ cells/mouse. The average number of N418⁺ cells obtained from lymph node cells after injection of adjuvant is 200 × 10⁵ mouse, corresponding to a 30-fold increase (data not shown).

BALB/c mice were therefore immunized with an irrelevant antigen (OVA) in IFA to induce cell recruitment in draining lymph nodes. Five days later, soluble HEL (100 nmoles/mouse) was injected i.v. and popliteal lymph nodes collected 3 h later. Amount and timing of soluble antigen injection were determined in pilot experiments (data not shown) and were found to be in agreement with previous results (3,5,18,19).

APC populations were enriched as in Fig. 1 and assessed in
relative role of DC and B cells in antigen presentation in vivo

Fig. 2. Time course of peptide-class II complex expression on lymph node DC and B cells following administration of protein antigen in
adjuvant. BALB/c mice were immunized into the hind footpads with 10 nmols/mouse HEL in IFA 3, 6 and 10 days before sacrifice. LNC from
5 (10 and 6 days) or 10 (3 days) mice were pooled and DC and B cell populations enriched as in Fig. 1. HEL108–116-specific I-Ed-restricted
T H111.3 T hybridoma cells were cultured (5×10^4 cells/well) with graded numbers of enriched APC populations. After 24 h, antigen specific
IL-2 production was determined using a two-site sandwich ELISA.

vitro for their antigen-presenting activity. Results in Fig. 3 demonstrate that HEL108–116/E^D complexes derived from
processing of HEL administered subcutaneously in IFA are only detectable on the DC-enriched population (Fig. 3A). Conversely, small and large B cells are much more efficient
than DC to activate T cell hybridomas when HEL is adminis-
tered in soluble form i.v. (Fig. 3B). To control for the intrinsic
capacity of DC and B cells to activate class II-restricted T
cells, we have analyzed presentation of naturally processed
self-β2M peptides corresponding to the sequences 26–39 by
I-A^D molecules (17). All three APC populations constitutively in presenting capacity:
DC-enriched > large B cells > small B cells. The intrinsic
capacity of these APC populations to present endogenously synthesized self-β2M peptide–A^D com-
plexes with the following hierarchy in presenting capacity:
DC-enriched > large B cells > small B cells. The intrinsic
capacity to present exogenous HEL is clearly different. These data demonstrate that the
mode of protein antigen administration determines preferential presentation of peptide-class II complexes by lymph node
DC or B cells.

Lymph node DC present in vitro to class II-restricted T cells
protein antigen much less efficiently than synthetic peptides
The previous results could be explained by differences in the
accessibility of circulating proteins to these APC populations,
or by their different potential in antigen uptake and processing
in situ. To address this point we have compared the capacity
of B cells and DC freshly isolated from immune lymph nodes
to present HEL or HEL peptide 105–120 in vitro (Figs 4 and 5). No qualitative differences are observed for B cells, either
small or large, in their capacity to present processed protein
or synthetic peptide. Conversely, DC are very efficient in
presenting the HEL peptide 105–120 but have an impaired
capacity to present this epitope after processing of the native
HEL protein. This is demonstrated either by titration of APC
using a fixed concentration of antigen (Fig. 4), or by titration
of antigen using a fixed concentration of APC (Fig. 5). In the
latter case, 10^5 DC and 3×10^5 small B cells/well were used
to obtain a comparable T cell activation. Small B cells were
chosen because they are more frequent than large B cells
and more active in antigen presentation after administration
of soluble HEL (Fig. 3B). The results indicate that, unlike B
cells, DC recruited in draining lymph nodes by s.c. adminis-
tration of adjuvant have a reduced ability to endocytose and
process protein antigens, while they remain extremely efficient
in presenting antigenic peptides, likely as a consequence of
their maturation in vivo. These data also show that for the
presentation of antigenic peptides DC are 10- to 30-fold more
potent APC as compared with large and small lymph node B
cells, respectively (Figs 4B and 5B).

Discussion
Data in the present paper demonstrate that different modes
of protein antigen administration lead to selective expression
of antigenic complexes by different APC (Fig. 6). Following
immunization with HEL in adjuvant, DC are the only lymph
node APC population expressing detectable HEL peptide–
class II complexes, whereas B cells are much more efficient
than DC in presenting antigenic complexes derived from
processing of circulating soluble HEL. To measure the capa-
city of resident lymph node APC (DC and B cells) to present
peptides derived from processing of a circulating protein
antigen, antigen has been injected shortly before lymph node
harvesting to minimize loading of APC before their entry into
the lymph node. Under these conditions, lymph node B cells
are far superior to DC in the presentation of HEL peptide–
class II complexes to T hybridoma cells. These data correlate
with the impaired capacity of lymph node DC to present
in vitro native protein antigen, as compared to synthetic
peptide, while no qualitative differences in the presentation
Relative role of DC and B cells in antigen presentation in vivo

Fig. 3. In vivo formation of antigenic complexes on lymph node DC and B cells following administration of protein antigen in adjuvant or in soluble form. BALB/c mice were immunized into the hind footpads with 10 nmol per mouse HEL in IFA (A and C) or 1 nmol per mouse OVA in IFA (B and D). OVA-IFA primed mice were injected with soluble HEL (100 nmol per mouse) 3 h before removal of popliteal lymph nodes. LNC from five mice per group were pooled and APC populations enriched as in Fig. 1. The HEL-specific I-E<sup>d</sup>-restricted 1H11.3 and the self B<sub>2</sub>M-specific I-A<sup>d</sup>-restricted 2G12.1 T cell hybridomas (upper and lower panels respectively) were cultured (5×10<sup>4</sup> cells/well) with graded numbers of enriched APC populations. After 24 h, antigen-specific IL-2 production was determined by adding 50 µl aliquots of culture supernatant to 10<sup>4</sup> CTLL cells for an additional 24 h. [3H]Thymidine (1 µCi/well) was added during the last 5 h of culture. Data are presented as mean thymidine incorporation (c.p.m.) from duplicate cultures. Background proliferation of CTLL was usually <1000 c.p.m. Results are from one representative experiment out of five performed.

Fig. 4. B cells present in vitro protein antigen more efficiently than lymph node DC. DC and B cells were obtained as in Fig. 2 from LNC of BALB/c mice primed with IFA alone 5 days earlier. The indicated numbers of APC enriched in DC (squares), large (triangles) or small (circles) B cells were cultured with the HEL<sub>108–116</sub>-specific, I-E<sup>d</sup>-restricted T cell hybridoma 1H11.3 in the presence of 1 µM HEL (A) or 0.5 µM HEL peptide 105–120 (B). After 24 h of culture, IL-2 concentration was determined using a two-site sandwich ELISA. Results are from one representative experiment out of three performed.
Relative role of DC and B cells in antigen presentation in vivo

Fig. 5. Protein antigen is presented in vitro more efficiently by small B cells as compared with immune lymph node DC. DC and small B cells were obtained as described in Fig. 1 from LNC of five mice primed with IFA alone 5 days earlier. 10^4 DC or 3 \times 10^5 small B cells/well were cultured with the HEL106–116-specific I-E^d-restricted T cell hybridoma 1H11.3 in the presence of the indicated concentrations of HEL (A) or HEL105–120 (B). After 24 h of culture, IL-2 production was determined using a two-site sandwich ELISA.

Due to the local secretion of proinflammatory cytokines such as tumor necrosis factor-\(\alpha\), which has been shown to downregulate the antigen-capturing and -processing capacity of human DC in vitro (21),

Since differences in antigen-presenting activity may also reflect differences in antigen uptake and processing capacity among the three APC populations tested, we analyzed in vitro the hierarchy in antigen-presenting activity to 1H11.3 T cells using synthetic peptide. As compared to large and small B cells, 10- to 30-fold less DC were needed to induce similar levels of IL-2 production by the T hybridoma cells. The number of antigenic complexes on DC required to activate T cells is much lower than for B cells (22). Thus, the more efficient presentation of soluble antigen administered i.v. by B cells rather than DC is likely to reflect a much higher expression of antigenic complexes on B cells as compared to DC, indicating that the circulating protein has been preferentially uptaken and processed in vivo by lymph node B cells. Interestingly, both resting and activated B cells, identified as DC and B cells in the draining lymph node. After administration of HEL in adjuvant DC are the only lymph node APC presenting HEL peptide–class II antigenic complexes. Conversely, after administration of soluble HEL i.v. B cells have a much more potent antigen-presenting activity than DC. These results indicate that DC but not B cells are the initiating APC in immune lymph nodes following administration of protein antigen in adjuvant. The high efficiency of DC in the presentation of antigenic peptides derived from proteins present in inflammatory sites coupled to the decreased capacity of DC recruited in immune lymph nodes to present soluble proteins is consistent with maturation of DC in vivo.

of protein and peptide are observed using B cells, in agreement with previous results (20). Even if we cannot formally exclude a possible inability of soluble antigen to access lymph node DC in vivo, the in vitro data are consistent with the interpretation that DC migrated to the lymph node have a reduced capacity to endocytose and present in situ soluble circulating protein antigen. Maturation of DC in vivo could be due to the local secretion of proinflammatory cytokines such as tumor necrosis factor-\(\alpha\), which has been shown to downregulate the antigen-capturing and -processing capacity of human DC in vitro (21).

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The different role of DC and B cells in the presentation of exogenous proteins in vivo exemplifies the necessity, by professional APC such as DC, to present selectively to lymph node T cells antigens available in inflammatory sites but not circulating soluble proteins. As it has been previously suggested (7), the inefficiency of mature DC to present soluble proteins, including circulating self antigens, to lymph node T cells may also provide an efficient mechanism to avoid self-antigen presentation by DC, thereby preventing the priming of potentially autoreactive T cells. On the other hand, once circulating antigen becomes available, lymph node B cells could further contribute to the clonal expansion of the antigen-specific T cells previously activated by DC (4,23,24), although this issue is still controversial (3). In this context, it is interesting to note that soluble antigen administration usually results in T cell tolerance, mediated by antigen presentation by B cells, rather than DC (25).

The fact that B cells are more efficient in presenting soluble
protein antigen as compared to DC seems to contradict previous results, where following protein administration i.v. splenic DC were the most important source of APC bearing antigenic epitopes (3,5). This could be explained by the use to monitor the formation of antigenic complexes, of antigen-specific T cell clones (5) or naive CD4+ T cell populations (3), which are probably more dependent on co-stimulation than T cell hybridomas. T1c clones proliferate optimally in response to splenic adherent cells rather than B cells (26). Similarly, it has been shown that DC are the most potent stimulators, as compared to B cells, to activate naive CD4+ T cells (27). Therefore, due to the APC requirement of such a read-out system, the antigenic complexes formed on B cells might have been underestimated. In addition, these experiments analyzed steady-state splenic APC isolated 2 h after i.v. injection of the protein antigen (5). It is therefore possible that the protein was preferentially taken up by resident immature DC, which frequency might be higher in a non-inflammatory situation like normal spleen, as compared to immune lymph nodes.

In conclusion, lymph node B cells, which are not able to present exogenous protein antigen administered s.c. in adjuvant, are nevertheless the most efficient lymph node APC in presenting circulating protein. Conversely, DC, probably as a consequence of their maturation process in vivo, are inefficient in presenting soluble protein antigen after migration to lymph nodes, but they present very efficiently antigenic peptides derived from processing of protein antigen endocytosed at the site of inflammation.

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Abbreviations

APC antigen-presenting cell
DC dendritic cell
HEL hen egg-white lysozyme
FA incomplete Freund’s adjuvant
LNC lymph node cells
β2M β2-microglobulin
OVA ovalbumin

References