Efficient Presentation of Multivalent Antigens Targeted to Various Cell Surface Molecules of Dendritic Cells and Surface Ig of Antigen-Specific B Cells[1]

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To study the relation between the form of an Ag and the response to it, we compared presentation in vitro with hen egg lysozyme (HEL)-specific T cells from TCR transgenic mice of free HEL and liposome-encapsulated HEL by different APC. HEL-specific splenic B cells or bone marrow-derived dendritic cells were incubated with free HEL or HEL-containing liposomes targeted by Ab to either surface Ig, the Fc receptor, or MHC class I and II molecules. Ag presentation by HEL-specific B cells was at least 100-fold more efficient for HEL in surface Ig-targeted liposomes than free HEL taken up by the same receptor or HEL in liposomes targeted to class I or II molecules. Ag presentation by dendritic cells from Fc receptor-targeted vesicles was augmented 1,000–10,000-fold compared with free Ag or nontargeted liposomes, but presentation was also efficient when Ag was targeted to class I or II molecules. These results indicate that Ag-specific B cells and dendritic cells can be equally efficient in stimulating IL-2 production by Ag-specific T cells from unimmunized TCR transgenic mice when the Ag is multivalent and taken up by appropriate receptors. In contrast to B cells, which require engagement of surface Ig for optimal presentation, dendritic cells may present Ag by means of several different cell surface molecules. ***The Journal of Immunology*****, 1998, 161: 6059–6067.**

B cells acquire Ag for presentation to T cells primarily via Ag-specific surface Ig (1). When Ags bear repeating determinants expressed at high density they can also activate specific B cells and may lead to differentiation and Ab production in a T-independent manner. Multivalent Ag determinants have been shown to be critical for stimulation of surface Ig of specific B cells, both in response to haptens coupled to lipid vesicles (liposomes) in the pioneering studies of Kinsky's group (2) and to membrane Ags of certain bacteria and viruses, in a T-independent manner (3). B cells can also take up and present T-dependent Ags encapsulated in liposomes (4) or associated with particles (5). Thus, surface Ig is responsible for both signaling and Ag acquisition. Although it has been reported that soluble hen egg lysozyme (HEL)[4] binding to surface Ig up-regulates costimulatory molecules on B cells (6), the potential role of Ag multivalence in activation of Ag-specific B cells with respect to their efficiency as APC for naive T cells has not been evaluated.

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[4] Abbreviations used in this paper: HEL, hen egg lysozyme; FcR, Fc receptor; PA-anti-DNP, protein A anti-dinitrophenyl Ab conjugate; CF, carboxyfluorescein.

Dendritic cells are thought to be the most efficient APC and as such are considered critical for the induction of immune responses (7). Lacking true Ag-specific receptors, immature dendritic cells can acquire Ags by fluid phase endocytosis and present them to T cells (8–10). This raises the question as to how an Ag is "perceived" by the dendritic cells in peripheral tissues, i.e., by what mechanisms Ag uptake induces the initiation of dendritic cell migration to secondary lymphoid organs (11) and up-regulation of costimulatory molecules (12, 13), which are events implicated in maturation of dendritic cells and necessary for their interaction with T cells. Dendritic cells express "pattern recognition receptors" (14), with affinity for repeating determinants, such as mannosyl groups, present on many bacteria (9, 15). These receptors clearly augment the efficiency of dendritic cells as APC for T cell stimulation, but the relative roles of Ag acquisition vs signals transmitted by these receptors have not been determined. Another strategy available for dendritic cells is to use the products of acquired immunity to recognize microorganisms expressing antigenic determinants identical to or cross-reactive with those for which Ab exists. The role of Fc receptor (FcR) in increasing the efficiency of presentation of opsonized Ag by dendritic cells has been reported (8, 16).

Both surface Ig and FcγRIII signal intracellularly use immunoreceptor tyrosine-associated motifs on cytoplasmic domains of the surface Ig-associated Igα- and Igβ-chains and the γ-chain of FcγRIII (17), or closely related sequences for FcγRII (18), suggesting analogous downstream events following receptor engagement. We therefore investigated the role of multivalent vs monovalent Ag targeted to surface Ig of B cells and the FcR of dendritic cells, with respect to the capacity of these cells to serve as APC to HEL-specific T cells from TCR transgenic mice. We compared these molecules with MHC class I and II determinants, which are present on both cells but are not known to be receptors implicated in the uptake of exogenous Ag.

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Table I. *Monoclonal Abs used in this study*

Antibody	Target	Isotype	Reference/Source
265.5	DNP	IgG1	Immunotech
U7.6.3	DNP	IgG1	Z. Eshhar, unpublished
U7.27.7	DNP	IgG2a	Z. Eshhar, unpublished
7.6 (anti-Id)	HEL-specific Ab (HH10)	IgG2a, κ	20
B1 23 2	HLA B and C	IgG2a, κ	50
H 100.5/28	H-2K^k α1 domain	IgG2a, κ	51
H 39.352.4	H-2 IE^k	IgG2a, κ	52
10-2-16	H-2 IA^k	IgG2a, κ	52
2.4G2	FcγRII, FcγRIII	Rat IgG2b	53

In this study we used HEL-containing liposomes with repeating hapten determinants on their surfaces. Results presented in this paper indicate that B cells transgenic for an anti-HEL surface Ig presented Ag much more efficiently when the protein was encapsulated in liposomes targeted to surface Ig than when HEL was free in solution. HEL encapsulated in liposomes opsonized by IgG Abs to the hapten is taken up and efficiently presented to T cells by dendritic cells via Ig FcR (FcγRII and/or FcγRIII), while HEL in nonopsonized liposomes was taken up and presented inefficiently. However, our results indicate that other cell surface molecules of dendritic cells, namely class I and class II MHC molecules, also permit efficient presentation of Ag in targeted liposomes. This is in contrast to the reduced capacity of these same molecules to serve an Ag-presenting function when B cells are used as APC. These results underline the different strategies of exclusive uptake by B cells of ligands corresponding to the specificity of their surface Ig as compared with less fastidious uptake by dendritic cells of particulate Ags with which they come into contact by means of a more varied group of cell surface molecules.

Materials and Methods

Mice

CBA/J mice transgenic for rearranged IgM and IgD Abs with variable regions derived from an Ab (HH10) with high affinity for HEL (19) were generated (20) using plasmids provided by A. Basten (Centenary Institute, Newtown, New South Wales, Australia). Characteristics of other transgenic lines made with these plasmids have been reported in detail (21). TCR transgenic mice (22), which express the same TCR as the 3A9 hybridoma (23), specific for the immunodominant HEL peptide 46–61 in the context of H-2 I-A^k, were kindly provided by M. Davis (Stanford University, Palo Alto, CA). Transgenic mice were maintained as heterozygotes backcrossed to CBA/J mice (IFFA-CREDO, L'Arbresle, France) and identified, by an ELISA for serum IgM anti-HEL for Ig transgenic mice (20) or by FACS analysis of TCR transgenic mice, as those in which a majority of peripheral blood CD4+ cells express Vβ8.2, the family of the gene used for construction of the strain (22). Mice used were between 6 and 12 wk of age.

Cells

Dendritic cells were obtained from femur bone marrow of normal CBA/J mice cultured in Iscove's modified Dulbecco's medium with 10% FCS, antibiotics, 2×10^{-5} M 2-ME, glutamine, and supernatants from NIH3T3 cells supplemented with 10–20 ng/ml murine granulocyte macrophage-CSF (24). After 3 days of culture, cells were diluted 1:1 in the same medium, and, after an additional 4–6 days of culture, plastic nonadherent cells were used as APC. At this time, the majority of these had immature dendritic cell morphology. B cells were purified by treatment of spleen cells with Abs to Thy-1, CD4 and CD8, and rabbit complement. T cells from the spleen were purified by passage over nylon wool columns, as described (25).

Antibodies

Abs used are described in Table I. The protein A-anti-DNP (PA-anti-DNP) conjugate was prepared by covalent coupling of Fab fragments of the mouse IgG1 anti-DNP mAb 265.5 (Immunotech, Marseille, France) to equimolar quantities of protein A (Pharmacia, Piscataway, NJ), modified with *N*-succinimidyl-6-maleimido caproate (Sigma, St. Louis, MO). The product was separated from multimeric conjugates and noncoupled species on Superdex 200 columns (Pharmacia). Monoclonal IgG1 and IgG2a anti-DNP Abs were produced from hybridoma cells kindly provided by Zelig Eshhar (Weizmann Institute of Science, Rehovot, Israel). All anti-DNP Abs were purified on protein G-Sepharose (Pharmacia). Ab 7.6 is an anti-Id (20) specific for HH10, the anti-HEL Ab expressed by the transgenic B cells used in this study. Other Abs were provided by the groups that published them or by the American Type Culture Collection (Manassas, VA).

Liposomes

Liposomes (80 μmol with respect to lipids) were made from 65% (mol/mol) dimyristoyl phosphatidylcholine and 34.5% cholesterol (both from Sigma) and 0.5% DNP-caproylphosphatidylethanolamine (Molecular Probes, Eugene, OR). Liposomes were formed by exposing lipids evaporated from chloroform/methanol (9:1 v/v) to an aqueous solution containing HEL (Sigma) (10 mg/ml; 700 μM) and 10 mM carboxyfluorescein (CF) (Molecular Probes) in PBS. For some experiments, liposomes of the same composition were made in CF/PBS or PBS alone. Following repeated cycles of freezing and thawing, liposomes were formed by extrusion (Extruder; Lipex Biomembranes, Vancouver, British Columbia, Canada) (26) through polycarbonate filters of 200-nm pore size (Corning Costar, Oneonta, NY) at 45°C, followed by gel filtration over Sepharose 4B columns (Pharmacia) to eliminate unencapsulated solute. Lipid vesicles were sterilized by filtration through 0.45 μm filters (Gelman, Ann Arbor, MI). Different HEL concentrations in lipid vesicles were obtained by dilution of this stock solution. HEL levels were confirmed by fluorescence of dilutions of free and liposome-entrapped CF with reference to the stock solution of HEL and CF used for liposome preparation. Use of ^{125}I-labeled HEL has confirmed that the fraction of encapsulated CF accurately reflects passive HEL entrapment (4). The internal volume of the liposomes used is about 4.2×10^{-18} liters, so at 700 μM Ag used, each liposome contains about 1750 HEL molecules.

Tests of Ag presentation

A schematic of the liposomes, the PA-anti-DNP conjugate, and the protein A-binding Ab that are used to target determinants other than the FcR is presented in Fig. 1*A*. Direct opsonization of the same liposomes by anti-DNP Abs, used to target the FcR, is presented in Fig. 1*B*. Twenty thousand (or as specified) B or dendritic cells were distributed in wells of 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) in RPMI 1640 supplemented with 5% FCS, 2×10^{-5} M 2-ME, glutamine, and antibiotics. Tenfold dilutions from the above stock solutions of free HEL or DNP liposomes containing HEL were added to the wells together with the PA-anti-DNP conjugate and/or Abs at a final concentration of 5 μg/ml. In some experiments, a washing step separated the exposure of cells to Ag and Abs or liposomes. Cells and Ags were incubated overnight at 37°C in a total volume of 100 μl, after which 20,000 T cells from transgenic or normal mice were added and incubated for an additional 48 h in a total volume of 110 μl. At this time, the concentration of IL-2 in supernatant fluids was determined by adding them to IL-2-dependent CTLL cells (10,000 cells/well). Following a 16-h incubation, proliferation of CTLL was assessed by measurement of radiolabeled thymidine incorporation into DNA at the end of an additional 8-h incubation in the presence of 0.5 μCi [^{3}H]thymidine. For studies of T cell proliferation, dendritic cells were irradiated (2000 rads) before Ag addition and [^{3}H]thymidine incorporation by T cells during an 8-h period measured following 96-h exposure to APC. All points are the means of duplicate determinations.

Flow cytofluorometry

Dendritic cells or B cells were incubated on ice with unlabeled or fluorescein-modified Abs (5 μg/ml), the PA-anti-DNP conjugate (5 μg/ml), and/or fluorescent or nonfluorescent liposomes corresponding to an HEL concentration of 100 nM before fixation. Analyses were performed on a FACScan using Lysis II and CellQuest software (Becton Dickinson, Mountain View, CA).

Results

Ag presentation by B cells is maximal for multivalent ligands targeted to surface Ig

Ag presentation to transgenic T cells was evaluated using B cells from anti-HEL transgenic or nontransgenic CBA/J mice as APC (Fig. 2). HEL was either free in solution or encapsulated in hapten-bearing liposomes. B cells from nontransgenic mice were unable to present Ag in either free or lipid vesicle-encapsulated form at HEL concentrations below 100 nM (1.5 μg/ml; Fig. 2*A*). As expected,

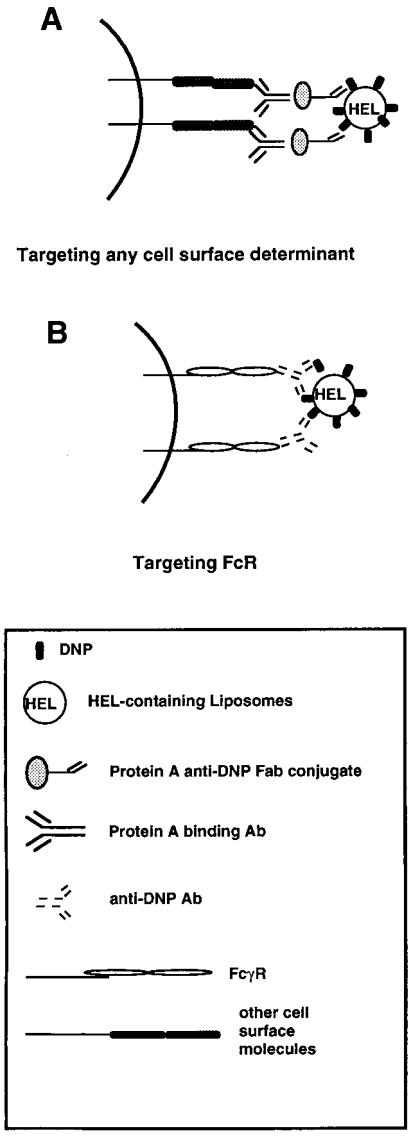

FIGURE 1. Schematic of the DNP-bearing liposome-Ab complexes used in this study. *A*, Targeting determinants other than the FcR with a PA-anti-DNP conjugate and protein A-binding Abs. *B*, Targeting the FcR by direct opsonization of the same liposomes with anti-DNP Abs.

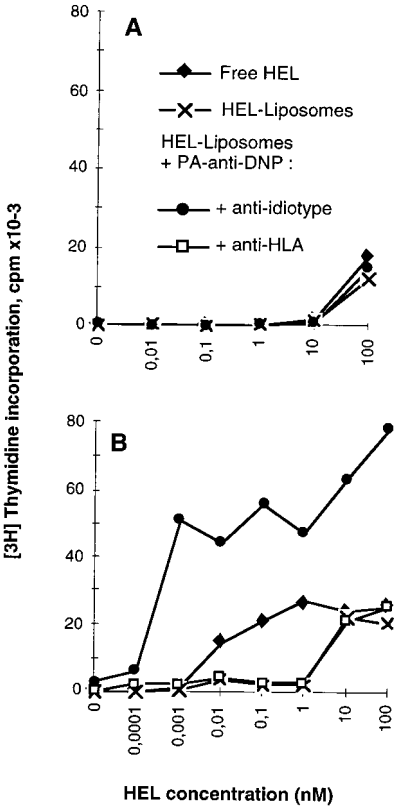

FIGURE 2. Advantage of multivalent Ag for presentation of HEL by transgenic B cells to transgenic T cells. Twenty thousand purified B cells from normal mice (*A*) or mice transgenic for anti-HEL Ig (*B*) were incubated overnight with various concentrations of HEL free in solution or encapsulated in DNP-bearing liposomes in the presence of a PA-anti-DNP Fab conjugate (5 μg/ml), together with an anti-Id Ab specific for the transgenic B cell receptor or an isotype-matched control Ab specific for HLA, at 5 μg/ml. Twenty thousand T cells from mice transgenic for an HEL-specific TCR were then added. The results correspond to proliferation of CTLL cells incubated in supernatants from duplicate wells obtained after an additional 48-h incubation of APC and T cells. These are data from an experiment representative of five performed.

B cells from anti-HEL Ig transgenic mice efficiently presented soluble HEL at concentrations as low as 10 pM (Fig. 2*B*). HEL encapsulated in liposomes was only slightly more efficiently presented by these transgenic mice than by nontransgenic mice in the absence of a cell-targeting Ab. Since HEL-specific B cells presented free HEL at 100-fold lower concentrations, this indicates that maximal leakage of HEL from lipid vesicles did not exceed 1% of the encapsulated Ag. Anti-hapten Fab coupled to protein A permitted targeting liposomes to surface IgM and IgD of Ig transgenic mice via the protein A binding anti-Id Ab 7.6 (20). In this situation, HEL in liposomes was presented at concentrations that never exceeded 1 pM HEL, a concentration 100-fold less than that required for presentation of free HEL directly taken up by the HEL-specific surface Ig and 100,000-fold less than free HEL or HEL in liposomes taken up by nonspecific B cells (Fig. 2*A*). The increased presentation of HEL in ligand-bearing liposomes, as compared with a monovalent HEL bound to the same receptor, could be related to increased delivery of the HEL into specialized intracellular compartments, up-regulation of costimulatory molecules due to the cross-linking of surface Ig, and/or an increased quantity of HEL in liposomes associated with the cells. Two series of experiments were performed to attempt to differentiate these possibilities.

To determine the effects of cross-linking of surface Ig, we incubated B cells in the cold with medium alone or with different concentrations of free HEL and then washed the cells. We then incubated them with the 7.6 anti-Id alone; with the 7.6 anti-Id and PA-anti-DNP complexes; or with the 7.6 anti-Id, PA-anti-DNP, and a fixed concentration of empty DNP-bearing liposomes overnight before the addition of transgenic T cells. The results are presented in Fig. 3. Responses to B cells incubated with HEL and then washed were comparable with responses without a washing step (data not shown). When cells were subsequently incubated with the 7.6 anti-Id a marked response was noted at an Ag concentration 100-fold lower than for cells incubated by Ag alone. This response was slightly increased by the addition of the PA-anti-DNP complex and was not further increased when DNP-bearing empty liposomes were added. No increased response was seen for liposomes in the presence of a control Ab, and no response at all seen for empty anti-Id targeted liposomes in the absence of free HEL. Thus, a fixed Ag dose was presented more efficiently when the surface Ig to which it bound was cross-linked. However, when

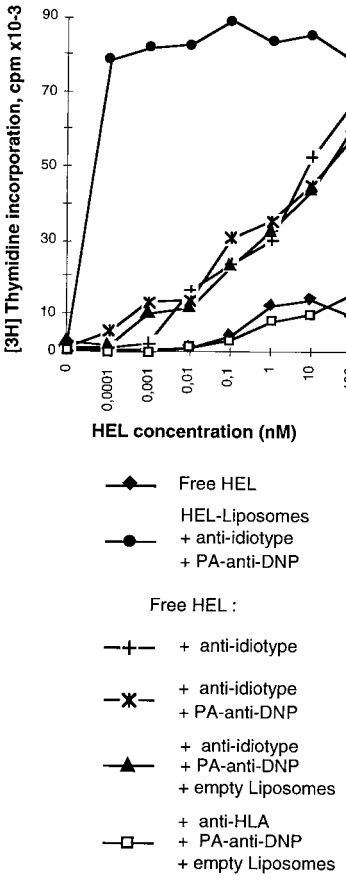

FIGURE 3. Effects of cross-linking surface Ig on Ag presentation. Twenty thousand B cells were incubated in the cold in medium alone or with various concentrations of free HEL, washed, and then incubated overnight with the 7.6 anti-Id alone (5 μg/ml), with the 7.6 anti-Id and PA-anti-DNP Fab conjugate (5 μg/ml), or with the 7.6 anti-Id, PA-anti-DNP Fab conjugate and a concentration of empty DNP-bearing liposomes identical to the number of liposomes sufficient to attain a concentration of 10 nM HEL, if the liposomes had contained Ag. Twenty thousand HEL-specific T cells were then added. The results correspond to proliferation of CTLL cells incubated in supernatants from duplicate wells obtained after an additional 48-h incubation of APC and T cells. These are data from one experiment representative of three performed.

targeted liposomes contained Ag the response was at plateau levels for Ag concentrations 100 times lower. Since induction of costimulation induced by cross-linking of surface Ig would be expected to be the same under conditions in which empty or HEL-containing liposomes were used, our results may be explained by a surface Ig-signaling-dependent modification of HEL presentation.

To evaluate the effects of the quantity of cell-associated Ag and the context in which it is perceived, we compared liposomes targeted to surface Ig by an anti-Id with protein A-binding Abs specific for other cell surface determinants. These were incubated with transgenic B cells in the presence of DNP-bearing liposomes and PA-anti-DNP. Incubation with HEL in liposomes targeted to either the MHC class II I-E^k molecule not involved in peptide presentation for this TCR or to the class I H-$2K^k$ molecule also augmented HEL presentation by 100-fold, i.e., to the same level as that observed for free HEL taken up via surface Ig, but at least 100-fold less efficient than Ag in liposomes targeted to surface Ig (Fig. 4*A*), while differences in binding of liposomes to these different determinants were minor (Fig. 4*B*). Ag targeted by an Ab to the I-A^k molecule was not presented, since this Ab is present in excess during the experiment and blocks recognition of the I-A^k-HEL peptide complex recognized by the TCR. Ag targeted to H-$2K^k$ or I-E^k was presented with the same efficiency whether transgenic or nontransgenic B cells were used (data not shown). Thus, the advantage for Ag presentation for HEL in liposomes targeted to surface Ig may not be explained only by increased Ag binding.

Ag presentation by dendritic cells when multivalent Ags are targeted to the IgG FcR

Dendritic cells are known to be excellent APC and are also known to have active fluid phase endocytosis (10). This endocytic activity is presumably related to the APC function, but few studies have compared presentation of Ag taken up by fluid phase or receptor-mediated processes. Dendritic cells were incubated with HEL or DNP-bearing liposomes in the absence of cell-targeting Abs or in the presence of IgG1 or IgG2a monoclonal anti-DNP Abs, together with HEL-specific T cells. Results for IgG1 are presented in Fig. 5*A*. Neither free HEL nor lipid vesicle-encapsulated HEL incubated with an irrelevant Ab were presented at concentrations below 10 nM. This minimum concentration is similar to those of published results for the presentation of free HEL by splenic dendritic cells to HEL-specific T hybridomas (27). In the presence of IgG1 or IgG2a anti-DNP Ab, DNP-bearing liposome-encapsulated HEL was as efficient for T cell stimulation at 10 pM, at least 1000-fold less than free HEL or nontargeted lipid vesicles. The anti-DNP Abs had no effect when HEL-containing liposomes were made without DNP on their membranes, or when DNP-bearing liposomes were made without encapsulated HEL (data not shown). The IgG FcR dependence of presentation mediated by the anti-DNP Ab was shown by the absence of efficient presentation in the presence of Fab (or $F(ab')_2$, data not shown) fragments of the IgG1 Ab and by inhibition of presentation of liposomes incubated with IgG1 (or IgG2a, not shown) by preincubation with the anti-FcγR Ab 2.4G2 (Fig. 5*B*). In parallel experiments, HEL in liposomes opsonized by intact anti-DNP Abs was not presented by B cells (data not shown), consistent with the inability of the B cell isoform of the FcR to take up Ag (28).

The ability of the anti-DNP Abs to mediate liposome-encapsulated Ag presentation by dendritic cells correlated with liposome binding to the cells. This depended on multivalent interaction with the Abs (Fig. 5*C*). Binding of fluorescence-labeled anti-DNP Abs was low in the absence of DNP-bearing liposomes. Their binding increased markedly in the presence of nonfluorescent DNP liposomes. The binding and presentation mediated by the 265.5 IgG1 Ab was similar to that of U7.6.3, an independently derived monoclonal IgG1 anti-DNP Ab (data not shown). Similar patterns of binding were seen when we used nonfluorescent anti-DNP Abs and DNP-bearing fluorescent liposomes (data not shown). Inhibition by 2.4G2 of binding of liposomes to the cells mediated by IgG1 and IgG2a Abs (Fig. 5) correlated with inhibition of Ag presentation via those isotypes (data not shown). Since Ab 2.4G2 is specific for FcγR II/III (29), all of the FcR-mediated binding and presentation are due to targeting to one or both of these receptors. The homologue of the high affinity (CD64) Ig FcR reported to be present on dendritic cells from human blood (30, 31) consequently does not appear to participate in the uptake of Ag by mouse dendritic cells derived from bone marrow under these culture conditions.

Ag presentation by dendritic cells is also efficient for multivalent ligands targeted to several cell surface determinants other than the FcR

The above results indicate that the FcR can play a role for dendritic cells analogous to that of surface Ig for B cells. In contrast to the

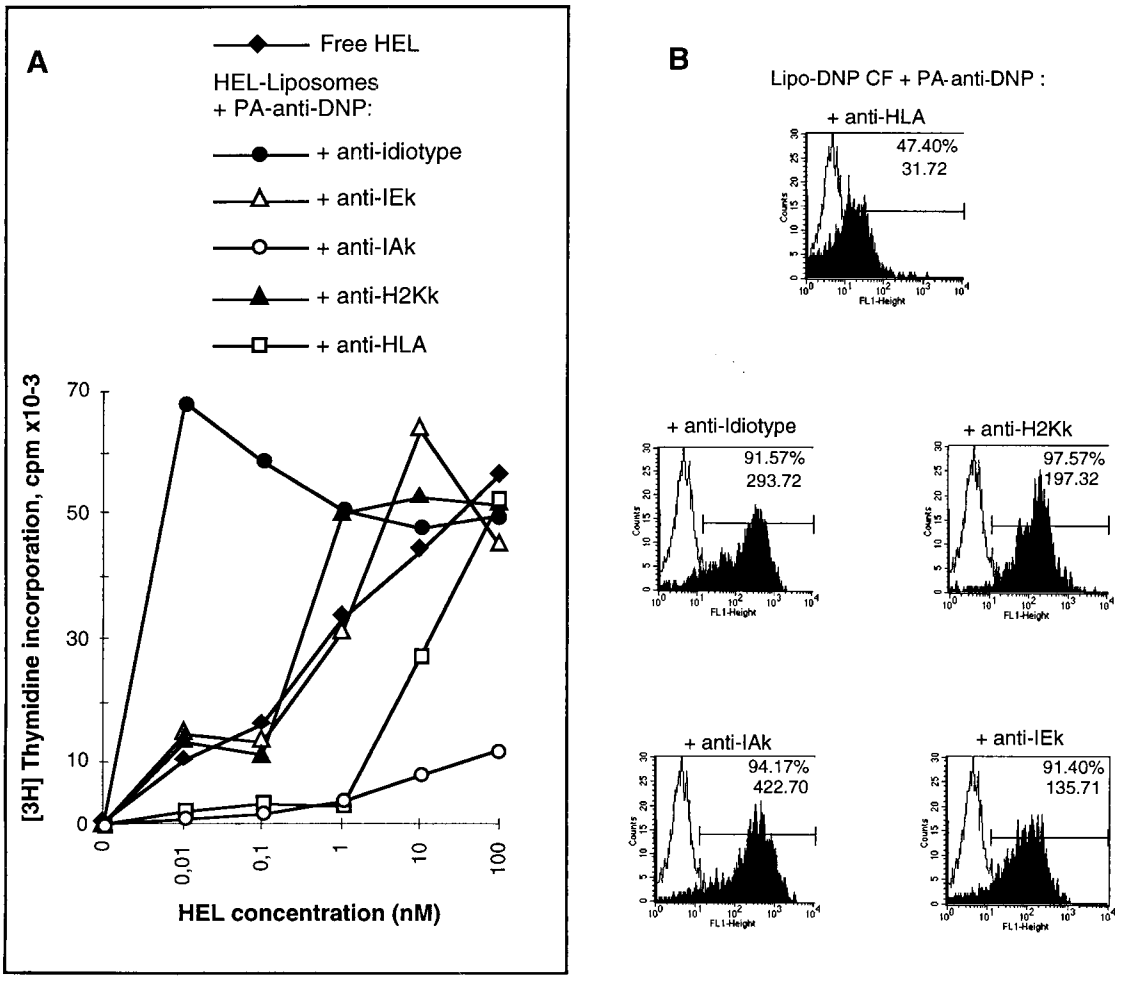

FIGURE 4. Multivalent Ag targeting to surface Ig of transgenic B cells generates the most efficient stimulus for IL-2 production by T cells. *A*, Purified B cells from mice transgenic for anti-HEL Ig were incubated overnight with various concentrations of HEL, free in solution or encapsulated in DNP-bearing liposomes together with an anti-Id Ab specific for the transgenic B cell receptor, or isotype-matched Abs specific for H-2K^k, I-E^k, I-A^k, or control HLA (5 μg/ml), in the presence of the PA-anti-DNP Fab conjugate (5 μg/ml). Transgenic T cells were added and assayed as in Fig. 2. These data are from a representative experiment of three. *B*, FACS profiles of purified transgenic B cells from the above mice alone (open silhouettes) or with DNP-bearing CF containing liposomes (Lipo-DNP CF) in the presence of a PA-anti-DNP Fab conjugate and the Abs specific for the molecules indicated in *A*. Profiles for 3000 cells are presented. The figures within the FACS profiles refer to the percentage of cells positive for the fixation of the liposomes and the mean fluorescence of that population.

situation for B cells, increased Ag presentation by dendritic cells was also seen when the PA-anti-DNP conjugate was used for targeting together with anti-class I (H-2K^k) or II (I-E^k) Abs (Fig. 6*B*); a separate experiment targeting I-E^k is presented in Fig. 6*A*. As for the situation with B cells, Ag presentation was not seen when the restricting I-A^k molecule was targeted (Fig. 6*B*). The anti-FcγR Ab 2.4G2 did not inhibit presentation of Ag delivered in the presence of protein A-bearing Abs (data not shown). Thus, dendritic cells are not efficient APC for HEL present free in solution or in neutral liposomes at biologically relevant concentrations (~150 ng/ml) but may present Ag to T cells about as efficiently as Ag-specific B cells (15–150 pg/ml), provided that the Ag makes multivalent contact with the cell. The same class I and II molecules that were inefficient for Ag presentation when expressed on B cells were more efficient when dendritic cells were targeted.

IL-2 production by transgenic T cells correlates with their proliferation

We asked whether the more efficient Ag presentation by dendritic cells for FcR targeted as compared with nontargeted Ag correlated with a more efficient Ag presentation by each cell, thus reducing the number of APC required. The number of dendritic cells necessary for the stimulation of IL-2 production by transgenic T cells was evaluated as a function of the mode of Ag acquisition. Variable numbers of irradiated dendritic cells were incubated with 10 nM free or 1 nM liposome-encapsulated Ag targeted by intact or Fab fragments of an IgG1 anti-DNP Ab. As few as 1000 dendritic cells generated a plateau level of IL-2 production when the FcR was targeted, whereas 10 times more dendritic cells were required for plateau stimulation of T cells at a 10-fold higher concentration of free Ag. Dendritic cells incubated under conditions of optimal Ag stimulation but in the absence of transgenic T cells failed to produce any factor capable of stimulating [^{3}H]thymidine incorporation by CTLL cells (Fig. 7*A*).

The production of IL-2 and the subsequent proliferation of T cells may be dissociated, if, for example, the amount of IL-2 produced is insufficient to support proliferation, or if the T cell is incompletely activated and does not up-regulate IL-2 receptors. However, our results demonstrate that the optimal conditions for induction of IL-2 production by responding transgenic T cells correlated with conditions for optimal stimulation of their directly

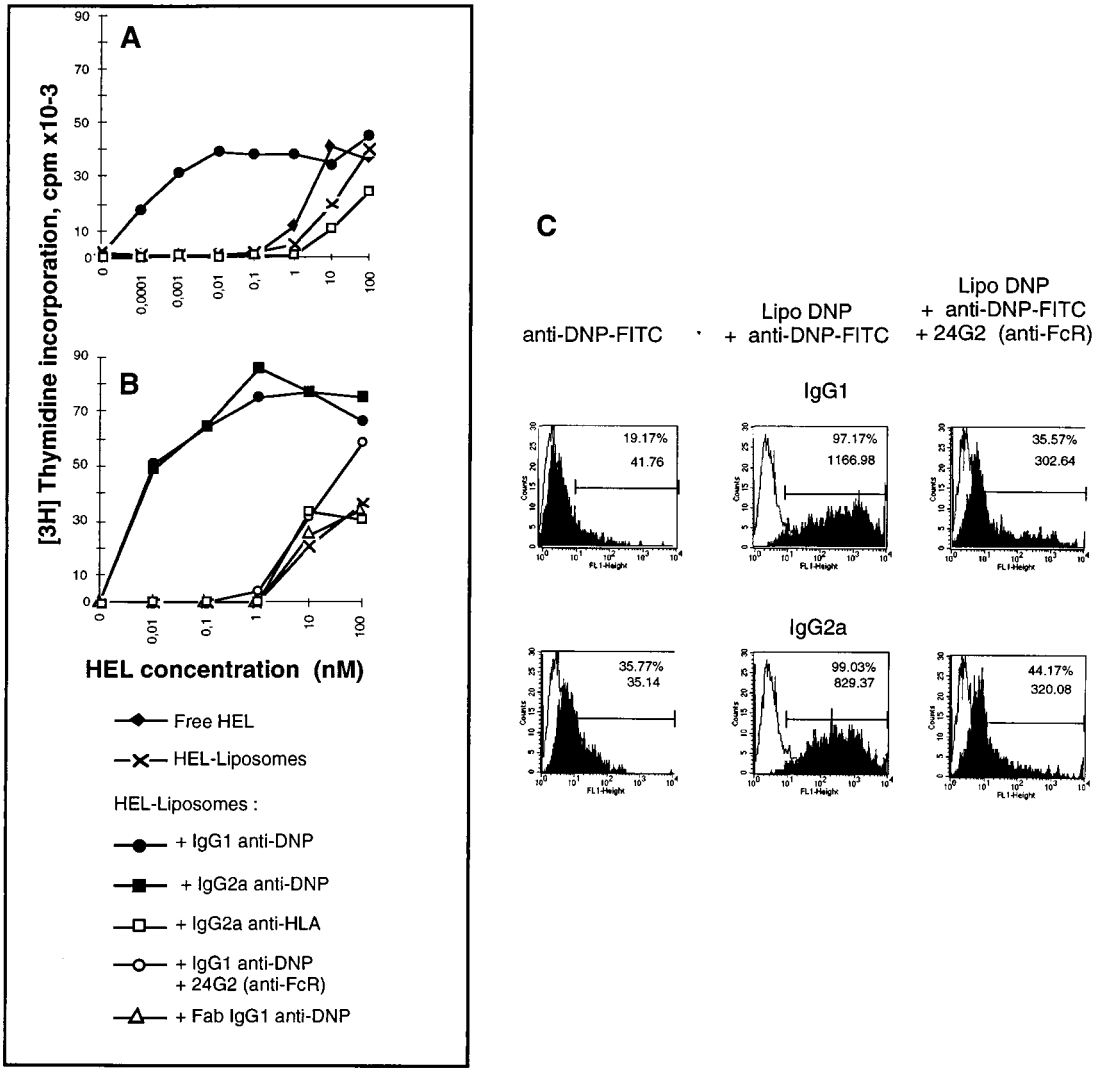

FIGURE 5. Efficiency of the IgG FcR for presentation of liposome-encapsulated Ag by dendritic cells. *A*, 15,000 dendritic cells obtained after culture of bone marrow for 7 days in the presence of granulocyte macrophage-CSF were incubated overnight with various concentrations of HEL, free in solution or encapsulated in DNP-bearing liposomes in the presence of anti-DNP Ab of the IgG1 isotype, or a control Ab specific for HLA at 5 μg/ml. T cells were added and assays were performed as described in Fig. 2. These are data from a representative experiment of more than 10 for IgG1 and free HEL. *B*, Dendritic cells as above were incubated overnight with various concentrations of HEL encapsulated in DNP-bearing liposomes with anti-DNP Abs of the IgG1 or IgG2a isotype, with anti-DNP Abs of the IgG1 isotype in the presence of the anti-FcR Ab 2.4G2 or in the presence of a Fab fragment of the IgG1 anti-DNP Ab. Data are representative of three experiments. *C*, FACS profiles of dendritic cells alone, with FITC-labeled Abs to DNP (5 μg/ml) alone, with DNP-bearing nonfluorescent liposomes (sufficient to contain 100 nM HEL) in the presence of FITC-labeled IgG1 or IgG2a DNP-specific Abs (5 μg/ml) to opsonize these liposomes, or with these liposomes and Abs together with the anti-FcR Ab 2.4G2 (20 μg/ml). Profiles for 3000 cells are presented. Data are representative of three analyses. The open silhouettes represent cells alone (*left*) or cells and FITC-modified anti-Id, as control (*middle* and *right*). Percentage of positive cells and mean fluorescence are indicated as for Fig. 3.

measured [^{3}H]thymidine incorporation after a period of culture extended to 4 days (Fig. 7*B*).

Discussion

We compared the presentation of soluble and liposome-encapsulated Ag by dendritic and B cells. DNP-bearing liposomes were opsonized by anti-DNP Abs for studies of interaction with Fc receptors (Fig. 1*B*). Alternatively, the use of these liposomes with PA-anti-DNP Fab conjugates permitted intact protein A-binding Abs to be oriented correctly toward other target molecules (Fig. 1*A*) while simultaneously blocking association with Fc receptors (32). Liposomes of the type used in this study are consequently models for infectious agents, which may interact with any of a number of cell surface determinants via multivalent ligands (13), and which contain multiple copies of Ag behind the barrier of a membrane or capsule. In the present experiments, each liposome expresses more than 1500 DNP molecules, permitting a high density of targeting ligands, based on DNP binding by intact anti-DNP Abs to dendritic cells or by PA-anti-DNP conjugates to other determinants on B cells or dendritic cells.

Differences in stimulation of Ag-specific T cells will depend on the consequences of interaction between Ag, or the liposome in which it is entrapped, and the particular APC surface molecule to which the Ag is targeted. These will be determined by 1) the density of the target molecules; 2) the signals generated by binding of Ag to the receptor; 3) the rate and extent of their endocytosis; 4) the quantity of the Ag delivered; and 5) the intracellular fate of the Ag, including degradation and access to class II molecules. Intact

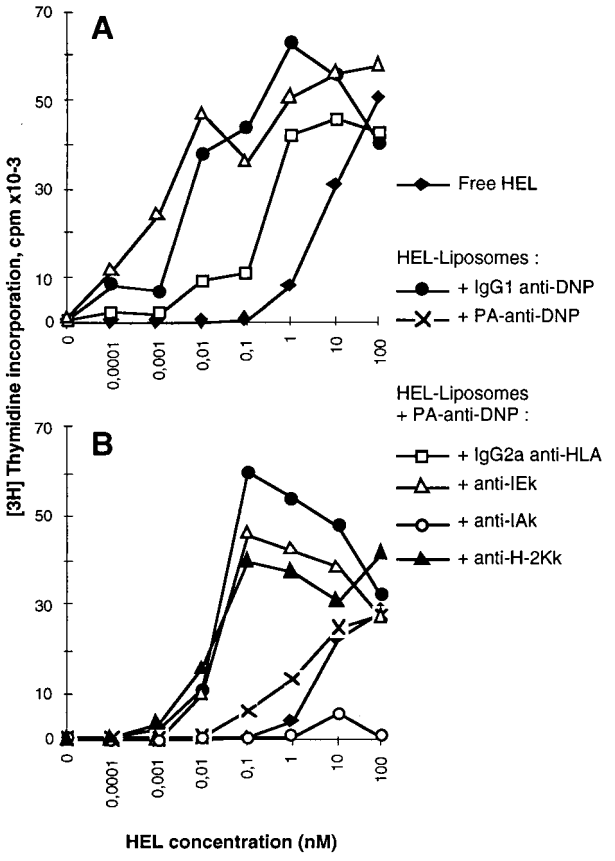

FIGURE 6. Multivalent targeting to several different dendritic cell surface molecules results in efficient T cell production of IL-2. *A*, Dendritic cells were incubated overnight with various concentrations of HEL, free in solution or encapsulated in DNP-bearing liposomes together with an IgG1 anti-DNP Ab, or with a PA-anti-DNP Fab conjugate and IgG2a Abs specific for H-2K^k, I-E^k, I-A^k, or control HLA. Transgenic T cells were added and assayed as in Fig. 2. Data from two representative experiments of four performed are presented. *B*, As in *A*, except that the control consists of HEL liposomes and the PA-anti-DNP Fab conjugate incubated in the absence of a targeting Ab.

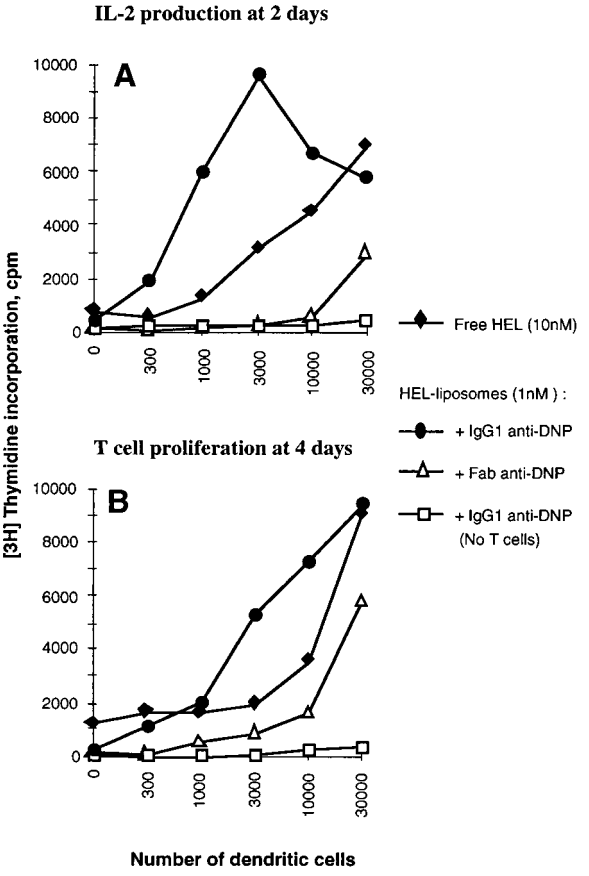

FIGURE 7. IL-2 production by transgenic T cells correlates with their proliferation. *A*, Various numbers of dendritic cells were incubated as in Fig. 4, except that they were irradiated (2000 rads) before exposure to Ag. They were incubated with HEL or HEL-containing, DNP-bearing liposomes at various concentrations together with IgG1 anti-DNP Ab or a Fab fragment of that Ab. T cells were not added to one series of dendritic cells incubated with liposomes and intact Ab. IL-2 production was evaluated by the incorporation of [^{3}H]thymidine by CTLL cells starting at 48 h after addition of T cells. *B*, For studies of proliferation, [^{3}H]thymidine incorporation by T cells (or of dendritic cells incubated alone) was measured directly after 96 h of exposure to APC.

monovalent Ag has been shown to be able to permit transgenic B cells to stimulate transgenic T cells bearing the same TCR as those used in the present study, at comparable Ag concentrations (6, 22). This monovalent form of Ag presentation by B cells is shown in the short term in vitro studies performed here to be much less efficient for stimulation than the same quantity of Ag bound to surface Ig that is subsequently cross-linked by Ab or empty liposomes. This difference for the same target molecule and APC is consistent with modulation of signals that are transmitted by surface Ig as a consequence of cross-linking, as has already been demonstrated in studies in which mutated Igα or Igβ molecules associated with surface Ig on B cells were transfected into myeloma or lymphoma cells (33, 34). However, cross-linking of surface Ig in the presence of a fixed amount of bound free HEL increased Ag presentation to a much lesser extent than when liposomes bound to surface Ig contained HEL (Fig. 3). HEL is passively entrapped within the aqueous spaces of these liposomes and presumably behaves like free HEL when released in cells. This is in contrast to experiments in which Ags have been covalently hapten modified for binding to anti-hapten surface Ig (35, 36) or directly bound to anti-Ag Abs for targeting purposes. These may alter Ag processing, or result in modification of their presentation, depending on the site on the Ag to which the Ab binds (37, 38). In other experiments we have studied Ag presentation to T hybridomas specific for the 46–61 determinant, which requires extensive processing for presentation and is associated primarily with newly synthesized class II molecules, as well as T hybridomas specific for peripheral peptides included within the sequence of HEL, which are presented by recycling class II molecules. These results indicate that HEL in liposomes behaves like free HEL when taken up by fluid phase endocytosis and shares the markedly enhanced presentation of HEL when targeted to surface Ig, whatever the peptide specificity of the hybridoma.[5] There is thus no evidence that a specialized processing compartment is required for release of liposome-entrapped Ag.

It has been estimated that fewer than 300 HEL peptide-IA^k complexes may activate class II- plus peptide-specific T cell hybridomas (39), whereas about 1 HEL molecule in 750 taken up by fluid phase endocytosis by lymphoma cells generated I-A^k-associated peptides recognized by a hybridoma with the same TCR as that expressed by our TCR transgenic T cells (40). Although naive

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transgenic T cells are reported to require a somewhat higher number of class II-peptide complexes for IL-2 production than primed T cells or hybridomas (41), the efficiency of class II association of Ag taken up by a receptor on APC may permit this level of peptide binding to be achieved at low external Ag concentrations, as suggested by the present studies. The efficiency of this process will also depend on the form of the Ag. Each liposome used here contains hundreds of HEL molecules, so that surface Ig targeted by anti-Id will internalize much more Ag than the two HEL molecules in free form taken up by the same receptor. Thus, the internalization of Ag in monovalent form could be considered as an "analogue" event, with the Ag dose internalized linearly with increasing numbers of receptors engaged, whereas internalization of multivalent Ag is a "quantal" event, in which one or only a few liposomes may contain sufficient Ag to induce a response, if delivered into a suitable compartment. This is an appropriate response in nature, in which quanta of Ag will normally be contained within infectious organisms. However, this sensitivity is manifest only in the context of cross-linking of surface Ig. Indeed, targeting of liposomes and the same PA-anti-DNP conjugates to I-E^k or H-$2K^k$, of which the density of expression on B cells is comparable with that of surface Ig, resulted in Ag presentation to a level not greater than that seen for the uptake of soluble HEL by anti-HEL surface Ig. This strongly suggests that binding of Ag to these molecules does not result in signaling that permits up-regulation of costimulatory molecules and access of Ag to class II-containing compartments. The recent development of Abs specific for HEL peptide-IA^k conjugates (40, 42) will permit quantification of the actual I-A^k association of Ag presented in monovalent or multivalent forms targeted to different cell surface molecules.

Several studies have emphasized the endocytic capacities of dendritic cells (9, 10). The liposomes we use are small enough to be taken up by fluid phase endocytosis. Free HEL and nontargeted liposome-encapsulated HEL were presented by dendritic cells equally well at equivalent Ag concentrations, ruling out any protective effect of the liposome membranes with respect to Ag processing. The presentation by dendritic cells of Ag associated with phagocytosis of micrometer-sized latex particles has been shown to be orders of magnitude more efficient than uptake of the same Ag in solution (43). Nonrecognition of the 200 nm DNP-bearing neutral liposomes used here in the absence of a targeting ligand permitted an unambiguous evaluation of the role of defined receptors in the uptake process. These lipid vesicles could be used to bind intact anti-DNP Ab, resulting in Ag presentation by IgG FcR-bearing dendritic cells. Stimulation of Ag-specific T cells by liposome-encapsulated HEL targeted to the FcR of dendritic cells by anti-DNP Abs required 1000-fold less Ag than when the cells were exposed to free HEL or nonopsonized liposomes containing HEL. Ag delivered by liposomes opsonized by PA-anti-DNP conjugates in the presence of Abs specific for class I or nonrestricting class II molecules on dendritic cells also resulted in presentation of Ag, resulting in IL-2 production at low Ag concentrations. This contrasts with the reduced efficacy of Ag presentation from liposomes targeted via class I and II molecules on B cells, as compared with surface Ig-targeted liposomes. The fact that we targeted identical class I and II molecules on B and dendritic cells rules out any possible effects of the affinity of receptor-ligand interactions on processing of the liposome-encapsulated Ag. On the other hand, this study compares B cells directly obtained from the spleen with dendritic cells maintained in culture. The phenotype of cultured dendritic cells may change rapidly, with down-regulation of Fc receptors and up-regulation of class II molecules, although the culture conditions used here are reported to maintain an immature phenotype (44). The respective roles of different dendritic cell surface molecules in Ag presentation in vivo remain to be tested.

B cells derived from mice in which the class II-associated invariant chain has been deleted cannot present class II-restricted Ag, while dendritic cells from the same mice present these Ags normally (24). These results indicate that Ag-processing pathways of dendritic and B cells differ. Recent studies indicate that cross-linking of surface Ig may alter intracellular transport of class II molecules and increase its association with endosomal compartments to which Ag is transported by surface Ig (45, 46). The experiments reported here suggest that Ags targeted to surface Ig on B cells are efficiently presented as a consequence of alteration of transport induced by Ig cross-linking. The FcR serves a similar role on dendritic cells, but its participation appears less predominant for dendritic cells than that of surface Ig for B cells. The function of dendritic cells as "sentinels" exposes them to infection by numerous microorganisms. This parasitism of APC is useful for host defense, provided that the act of entering the dendritic cell results in T cell activation. The capacity of dendritic cells to present Ags that cross-link molecules, including MHC class I and II molecules that are not nominal cell surface Ag receptors, may counter the potential use of these molecules as receptors by microorganisms. While the presence of immunoreceptor tyrosine-associated motifs in molecules associated with both surface Ig on B cells and the FcR of dendritic cells suggests common downstream pathways potentially responsible for both efficient presentation and activation (47), the mechanisms permitting Ag presentation or activation via other molecules on dendritic cells remain to be determined.

The question as to whether B cells can initiate immune responses, or are better or worse as APC for T cells than dendritic cells, has been long posed. Early studies indicating that B cells cannot initiate responses are based on experiments in which surface Ig is not stimulated at all, as when exogenous or constitutive peptides are presented (48, 49). Subsequent studies indicated that this presentation defect could be partially overcome by monovalent Ag (22). In the present study the efficiency of Ag presentation by B cells the surface Ig of which was targeted by multivalent Ag was further augmented by orders of magnitude. Furthermore, our data indicate that contact with multivalent Ag may be as important for Ag presentation by dendritic cells as it is for Ag-specific B cells. Thus, these results suggest that when access to Ag is assured, Ag-specific B cells and dendritic cells may have equivalent capacity for Ag presentation to Ag-specific $CD4^+$ T cells. In fact, presentation of Ag from relevant pathogens may be a cooperative process between B cells and dendritic cells under physiologic conditions, since by secreting Ab into the circulation, B cells can permit dendritic cells to identify Ag via their Fc receptors. The requirement that Ab pre-exist to promote processes that augment production of Abs of equivalent specificity does not necessarily pose a dilemma because of the neonatal availability of IgG Ab of maternal origin, as also suggested by others (31). Maternal Ab will permit dendritic cells to present environmental Ags, increasing the frequency of Ag-specific T cells and compensating for the rarity of Ag-specific B cells.

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