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Presentation of Exogenous Antigen with Class I Major Histocompatibility Complex Molecules

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Soluble antigens (Ags) in the extracellular fluids are excluded from the class I major histocompatibility complex (MHC)–restricted pathway of Ag presentation in most cells. However, an exogenous Ag can be internalized, processed, and presented in association with class I MHC molecules on specialized Ag-presenting cells (APCs). These APCs express class II molecules and can simultaneously present exogenous Ags to both class I and class II MHC–restricted T cells. These APCs may be important participants in the regulation of host immune responses. This APC activity may explain several phenomena of cytotoxic T lymphocyte (CTL) priming in vivo and might be exploited for eliciting CTL responses to protein vaccines.

ANTIGENS IN THE EXTRACELLULAR fluid are taken up by specialized APCs, processed in an endosomal compartment, and subsequently displayed in association with class II MHC molecules (*1*). In contrast, endogenously synthesized Ags, of virtually all cells, are processed in a distinct intracellular compartment and are subsequently displayed in association with class I MHC molecules (*2, 3*). These two pathways for Ag processing and presentation are segregated, which influences the specificity of immune responses (*3–5*). This segregation helps determine the host response to a particular pathogen. For example, as a consequence of the segregated pathways, cytolytic responses are selectively targeted to virally infected or transformed cells. However, if there is absolute segregation of class I and class II MHC Ag-processing pathways, it is then unclear how the necessary collaboration between T helper cells and CTLs occurs. Previous studies have analyzed Ag presentation with APCs in conventional cytolytic assay systems. We have used a novel, class I MHC–restricted T-T hybridoma (*6*) to examine the segregation of class I and II MHC–restricted pathways of Ag presentation in APCs that reside in normal lymphoid organs.

The ability of various APCs to present an exogenous protein Ag to T cells was ana-

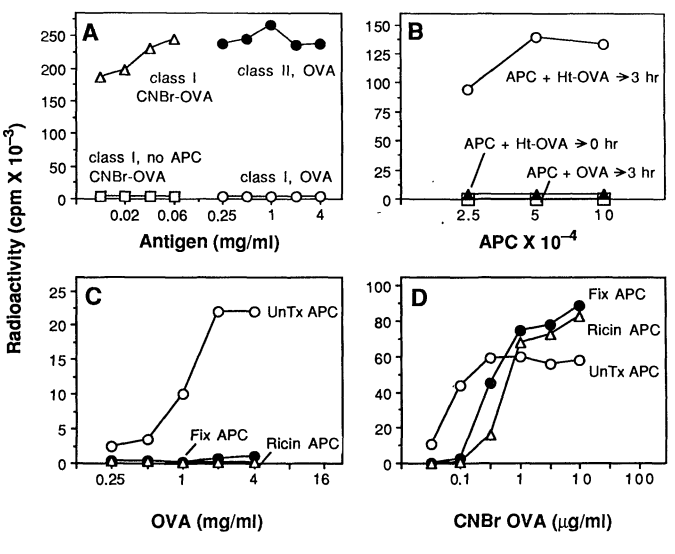

Fig. 1. Presentation of OVA by different APCs. (A), (B), (C), and (D) were assayed with T cells that are class I restricted. Some assays in (A) were also done with T cells that are class II restricted. (**A**) Class I (10^5, RF33.70) (*6*) or class II (5×10^4, DO.11.10) (*16*) MHC–restricted T-T hybridomas were cultured in the presence or absence of untreated LB27.4 APCs (5×10^4) (*17*) with native OVA or cyanogen bromide–cleaved OVA (CNBr-OVA) as described (*18*). After 18 hours of incubation at 37°C, a 100-μl aliquot of supernatant was removed from duplicate cultures and assayed for IL-2 content with HT-2 cells (*19*). (**B**) Similar to (A), except that LB27.4 APCs were incubated for 10 min at 37°C with native OVA (10 mg/ml) under isotonic or hypertonic (Ht) conditions as previously described (*5*). Cells exposed to Ht conditions were diluted in hypotonic media and incubated for 3 min at 37°C to cause osmotic lysis of pinosomes, as described (*5, 7*). After several washes at 4°C the APCs were either fixed with paraformaldehyde (*20*) immediately or after 3 hours of incubation at 37°C. The number of LB27.4 cells was titrated in cultures with RF33.70 cells in the absence of exogenous Ag. (**C**) Similar to (A) but APCs were C57BL/6 splenocytes (10^6) that were either untreated (UnTx), fixed (Fix) with glutaraldehyde (*21*), or incubated with ricin (2×10^{-9} M) for 1 hour at 37°C and washed, and the Ag added to cultures was OVA. (**D**) Similar to (C) but the Ag added to cultures was CNBr-OVA. Data points are expressed as the mean incorporation of [^{3}H]thymidine (counts per minute) into HT-2 cells. Data are representative of at least three experiments.

lyzed with the use of T-T hybridomas that produce the lymphokine interleukin-2 (IL-2) upon recognition of ovalbumin (OVA) in association with class I (*6*) or class II MHC molecules. The OVA-specific, K^b-restricted T-T hybridoma, RF33.70, is not stimulated by the B lymphoblastoid APC, LB27.4, in the presence of exogenous, native OVA, even at high Ag concentrations (Fig. 1A). In contrast, LB27.4 cells presented OVA to the OVA-specific, class II MHC–restricted T-T hybridoma, DO.11.10 (Fig 1A), as expected.

LB27.4 cells presented exogenously added CNBr-cleaved OVA peptides in association with class I molecules (Fig. 1A). Similarly, when native OVA was introduced into the cytoplasm of LB27.4 by osmotic lysis of pinosomes (*5, 7*), this APC stimulated RF33.70 cells to produce IL-2 (Fig. 1B). LB27.4 APCs incubated with OVA under isotonic conditions did not stimulate RF33.70 cells (Fig. 1B). After osmotic Ag loading, the presentation of OVA by LB27.4 cells was initially inhibited by, and later resistant to, chemical fixation (Fig. 1B). These results probably indicate a requirement for Ag processing (*8*). Identical results were obtained in experiments with EL4 APCs (*9*). These and previous results (*5*) demonstrate that the route of entry of OVA into LB27.4 or EL4 APCs determines whether the Ag will be presented in association with class I MHC molecules.

In contrast, exogenous OVA was presented by normal splenocytes to RF33.70 cells (Fig. 1C). Similarly, splenic APCs that were previously incubated with exogenous, native OVA for 2 hours stimulated RF33.70 cells (Fig. 2A). Thus, splenic APCs can present exogenous OVA in association with class I MHC molecules.

This ability to present native OVA was lost when the splenic APCs were chemically fixed (Fig. 1C), or treated with ricin (Fig. 1C) or azide (Fig. 2A). Ricin and azide inhibit protein synthesis and cellular respiration, respectively. In contrast, glutaraldehyde-, ricin-, or azide-treated APCs retain the ability to present exogenously added OVA peptides in association with K^b (Figs. 1D and 2C). Although azide inhibited splenocyte uptake and presentation of exogenous native OVA, it did not affect the presentation of Ag that was previously associated with K^b molecules (Fig. 2B). Therefore, the various inhibitors did not affect the function of class I MHC molecules on the APC surface, or the ability of the APCs to interact effectively with T cells.

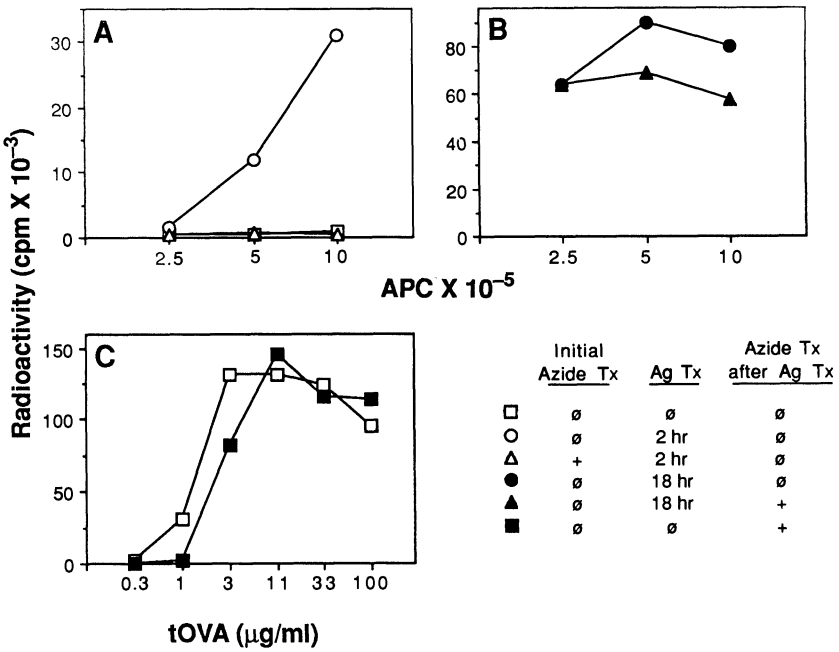

Fig. 2. Effect of azide on the presentation by splenic APCs of OVA in association with class I MHC molecules. (**A**) C57BL/6 splenocytes (20×10^7/ml) were incubated in the presence [(azide Tx) with +] or absence of azide (15 mM) for 3 hours at 37°C and then washed; where indicated, OVA (15 mg/ml) was added for the final 2 hours of incubation. The treated splenic APCs were added to cultures with RF33.70 cells. (**B**) Similar to (A) but splenocytes (10^7/ml) were incubated with a tryptic digest of OVA (tOVA) (300 μg/ml) in media (10% fetal bovine serum) for 18 hours at 37°C followed by 3 hours of incubation in the presence or absence of azide (15 mM) and washed. (**C**) Similar to (A) but tOVA was added to cultures of treated splenic APCs (10^6) and RF33.70 cells. Cultures were prepared, handled and assayed as described in Fig. 1. Data are representative of three experiments.

It was formally possible that OVA was processed in the extracellular fluid (*5*), by proteolytic enzymes secreted by the APCs. To examine this issue, we tested whether native OVA, incubated with splenocytes for 18 hours could associate with class I MHC molecules on the APC surface. Chemically fixed (Fig. 3A) or ricin-treated (*9*) APCs could not present the "splenocyte-conditioned" OVA (OVA-C.M.), but did present proteolytically cleaved OVA. Live splenocytes present OVA and OVA-C.M. with similar efficiency (Fig. 3A). In this experiment, the APCs that were previously incubated with the OVA were actively processing Ag, because they were subsequently capable of stimulating RF33.70 cells (see legend for Fig. 3).

In a second experimental protocol, we tested whether cocultures of live H-2^d APCs and fixed H-2^b APCs would stimulate RF33.70 cells in the presence of exogenous OVA. RF33.70 cells do not recognize OVA in association with H-2^d APCs and are not alloreactive to the *H-2*^{d} haplotype (*6*) (Fig. 3B). Live H-2^d splenocytes did not alter OVA in the extracellular fluid in a manner that allowed association with K^b on chemically fixed APCs (Fig. 3B). The fixed H-2^b APCs were competent to present exogenously added OVA peptides (Fig. 3). The live H-2^d splenocytes actively processed and presented OVA to the DO.11.10 T-T hybridoma (Fig. 3C), which served as a control for their functional competence. Together, these results indicate that under our experimental conditions, splenic APCs do not process OVA in the extracellular fluid at a detectable level. We conclude that there is a splenic APC capable of taking up exogenous OVA, processing the Ag in an intracellular compartment, and then displaying the processed Ag in association with class I MHC molecules on the cell surface.

It was of interest to determine whether this ability to take up exogenous Ags and present them in association with class I versus class II MHC molecules was the property of the same or a distinct population of APCs. Splenocytes that were depleted of conventional class II MHC–bearing accessory cells essentially did not present exogenously added OVA to either the class I or class II MHC–restricted T-T hybridomas, RF33.70 (Fig. 4A) or DO.11.10 (Fig. 4B), respectively. Splenocytes depleted of class II MHC–bearing APCs could still present exogenously added OVA peptides to RF33.70 cells (*9*). In contrast, splenocytes depleted of

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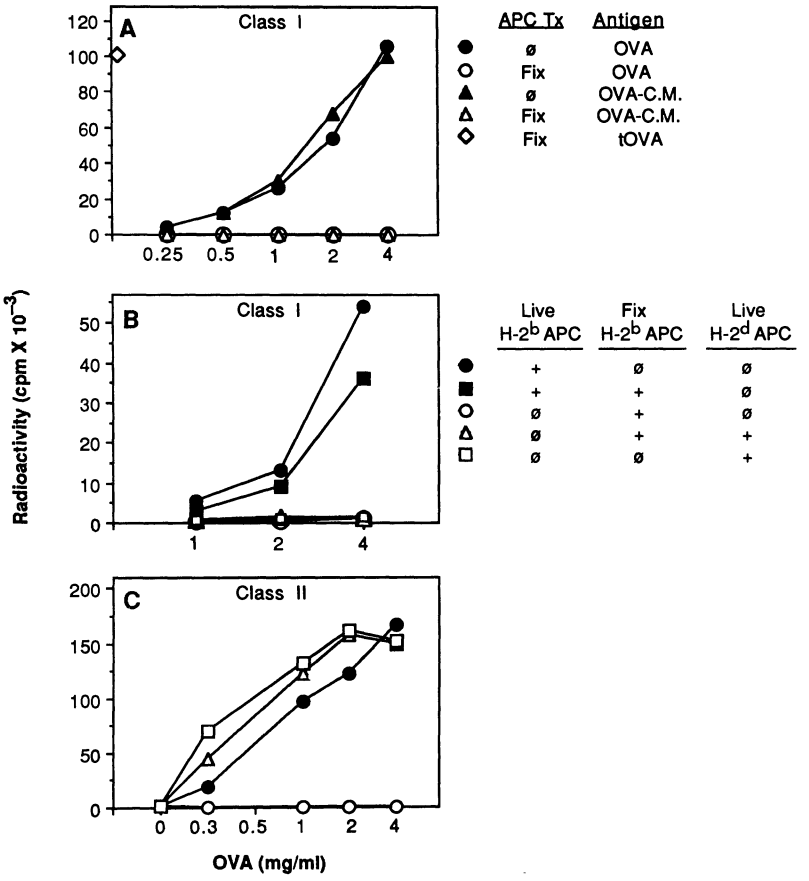

Fig. 3. Extracellular Ag processing is not detectable. (**A**) RF33.70 cells were cultured with untreated or glutaraldehyde-fixed C57BL/6 splenocytes (10^6) and tOVA (100 μg/ml), native OVA, or OVA-conditioned media (OVA-C.M.). OVA was incubated with C57BL/6 splenocytes (10^6/ml) in media for 18 hours at 37°C, cells were pelleted, and the culture supernatant was used as a source of OVA-C.M. The splenocytes used in the latter incubation had processed OVA as they stimulated RF33.70 cells (87×10^3 cpm). (**B**) RF33.70 cells were cultured with OVA, and the indicated mixture of untreated or glutaraldehyde-fixed splenocytes of C57BL/6 (*H-2^b*) and BALB/c (*H-2^d*) origin. The addition of tOVA (1 μg/ml) to cultures of fixed H-2^b splenocytes and RF33.70 cells resulted in 106×10^6 cpm in the IL-2 assay. (**C**) Similar to (B) but with DO.11.10 rather than RF33.70 cells. DO.11.10 cells recognize OVA in association with both I-A^d and I-A^b (*16*). Cultures were prepared, handled, and assayed as described in Fig. 1. Data are representative of three experiments.

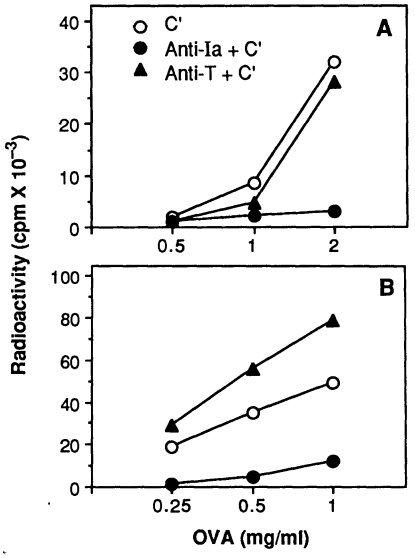

Fig. 4. Phenotype of splenic APCs presenting exogenous OVA. (**A**) RF33.70 cells were cultured with treated C57BL/6 splenic APCs (10^6) and OVA. Splenocytes were treated with rabbit complement alone (C'), monoclonal antibodies (MAbs) to Ia antigens (anti-Ia) [M5/114 (*22*) and 3JP (*23*)] and complement or MAbs to T cells (anti-T) [anti-Thy-1, M5/49 (*24*); anti-CD4, GK1.5 (*25*); and anti-CD8, HO2.2ADH4 (*26*)] and complement, as previously described (*27*). (**B**) Similar to (A) but with DO.11.10 rather than RF33.70 cells. Cultures were prepared, handled and assayed as described in Fig. 1. Data are representative of three experiments.

T lymphocytes presented exogenous OVA in association with both classes of MHC molecules. Therefore, the ability to take up and process exogenous OVA for presentation in association with class I MHC molcules is found only in a subset of normal splenocytes that expresses class II MHC molecules [Fig. 4 and (*9*)]. This APC should therefore simultaneously display processed OVA peptide in association with class I and II MHC molecules. Formal proof that a single cell can mediate both forms of Ag presentation would require the analysis of cloned APCs. These results imply that there are differences in the trafficking of Ags or class I MHC molecules in distinct APCs.

Previous investigations have analyzed only those APCs that are amenable for use as target cells in cytolytic assays. With these APCs, most exogenous Ags, including OVA (*3–5*), do not get processed and presented in association with class I MHC molecules. The few exogenous Ags that gain access to the class I MHC–restricted pathway of Ag presentation are thought to do so by virtue of a unique ability to enter the cytoplasmic compartment of APCs (*10, 11*). We confirm that a soluble exogenous Ag does not get processed and presented in association with class I MHC molecules on many cell types.

Exogenous Ags, in some cases, can gain access to the class I MHC–restricted pathway of Ag presentation in vivo (*12–15*). On the basis of this indirect evidence, an APC that can process and present exogenous Ags in association with class I MHC molecules has been postulated to exist (*12, 15*). However, alternative mechanisms such as extracellular proteolysis, could also account for these phenomena. Our results lend direct support to the former possibility and provide a mechanism to explain the above in vivo phenomena. These APCs may allow the priming of CTL responses with exogenous Ags under the appropriate conditions. Accordingly, the present findings and assay system may be useful for the development of vaccines.

We can only speculate on the physiological role and consequences of the APC activity that we have detected. A cell that expresses both class I and II MHC molecules and that can sample, process, and present Ags from both the extracellular and intracellular environments would be in a position to promote the interaction between T helper cells and CTLs. This could be of importance for T-T collaboration in general or in cases where the expression of an Ag is limited to class II–negative, nonlymphoid tissues, such as in a tissue-specific viral infection. It is also possible that the APC itself is affected by interaction with a CTL, which could account for some previous reports of antigen-specific suppression mediated by T cells.

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The Role of B Cells for in Vivo T Cell Responses to a Friend Virus-Induced Leukemia

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B cells can function as antigen-presenting cells and accessory cells for T cell responses. This study evaluated the role of B cells in the induction of protective T cell immunity to a Friend murine leukemia virus (F-MuLV)–induced leukemia (FBL). B cell–deficient mice exhibited significantly reduced tumor-specific CD4$^+$ helper and CD8$^+$ cytotoxic T cell responses after priming with FBL or a recombinant vaccinia virus containing F-MuLV antigens. Moreover, these mice had diminished T cell responses to the vaccinia viral antigens. Tumor-primed T cells transferred into B cell–deficient mice effectively eradicated disseminated FBL. Thus, B cells appear necessary for efficient priming but not expression of tumor and viral T cell immunity.

B CELLS ARE PART OF A HETEROGENEOUS population of antigen-presenting cells (APCs) that can activate CD4$^+$ helper T cells (*1*). The role of B cells in the induction and expression of in vivo T cell responses has been previously evaluated by rendering mice deficient in B cells by treatment from birth with high doses of rabbit antibody to mouse immunoglobulin M (IgM) (anti-μ) (*2*). These B-cell–deficient mice do not generate antigen-specific T cell responses after in vivo priming with hapten, a defect reflecting the requirement for B cells to function in vivo as APCs for T cells (*3*). The role of B cells in the in vivo presentation of larger, more complex antigens and the induction of CD4$^+$ helper and CD8$^+$ cytotoxic T cells is less clear. However, in vitro analysis of the response to large proteins such as thyroglobulin and vesicular stomatitis virus has demonstrated efficient in vitro activation of primed T cells by B cells, suggesting that B cell–APCs can potentially contribute to priming to large protein and viral antigens in vivo (*4*).

B cells could be particularly important for the induction of T cell responses to tumor cells, since limitations in antigen presentation could result from deficiencies in APC number and function that occur secondarily to progressive tumor growth or therapy (*5*). We have previously evaluated the T cell responses of C57BL/6 (B6) mice to FBL, a Friend murine leukemia virus (F-MuLV)–induced leukemia that expresses retrovirally encoded antigens and only class I major histocompatibility complex (MHC) antigens, but induces both CD4$^+$ and CD8$^+$ T cell responses (*6, 7*). The CD4$^+$ T cell response requires APC-expressing class II MHC antigens to process and present FBL, includes T cells that produce both interleukin-2 and interleukin-4 (IL-2 and IL-4), and is primarily directed at F-MuLV envelope epitopes. The cytolytic CD8$^+$ T cell response includes IL-2–dependent and IL-2–producing T cells that predominantly recognize F-MuLV Gag epitopes (*6, 7*). Adoptive transfer of FBL-specific T cells can completely eradicate disseminated FBL (*6, 8–10*). This FBL tumor model, with well-characterized T cell responses to tumor-associated retroviral antigens, was used to evaluate the role of B cells in the induction and expression of T cell responses to a tumor. The results demonstrate that B cells can play an essential role in the induction of in vivo T cell responses to retrovirally induced tumor cells.

Anti-μ–treated B6 mice were injected with FBL tumor cells, and the participation of B cells during in vivo priming was evaluated by measuring in vitro secondary T cell responses to FBL (*7*). B cell depletion was complete and selective; no IgM$^+$ cells or serum IgM could be detected, the B cell mitogen lipopolysaccharide (LPS) elicited no proliferative response, whereas the T cell mitogen conconavalin A (Con A) elicited a normal response (*11*). After immunization with FBL, the proliferative response to FBL of splenic T cells from anti-μ–treated mice was 50% that of control mice (Fig. 1), and essentially no response was detected in lymph node cells. These data, consistent

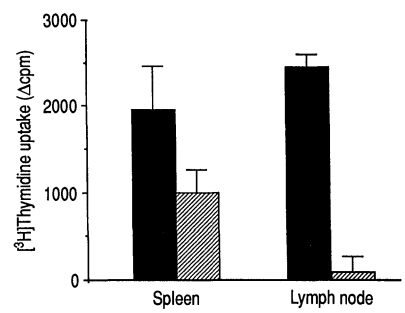

Fig. 1. Contribution of B cells to primary FBL-specific proliferative T cell responses. Spleen or lymph node cells were obtained from B6 mice (*H-2^b*) (Jackson Laboratory, Bar Harbor, Maine) treated from birth with rabbit IgG (solid bars) or anti-μ (hatched bars) and primed intraperitoneally at 6 to 9 weeks of age in vivo with 10^7 irradiated FBL. Responder cells (5×10^5), obtained 6 weeks after priming, and irradiated stimulator cells were cultured in 96-well, flat-bottom plates in RPMI 1640 containing 10% fetal bovine serum (FBS), 2.5×10^5 M 2-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 mg/ml), and L-glutamine. Cells were stimulated in vitro for 72 hours at a responder to stimulator ratio of 100:1 and assayed for incorporation of [^{3}H]thymidine. Data are presented as the mean difference with standard error bars in [^{3}H]thymidine uptake in FBL-stimulated and unstimulated cultures and represent one of three experiments.

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