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Intracellular Processing of Liposome-Encapsulated Antigens by Macrophages Depends upon the Antigen

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Two proteins, a recombinant malaria protein (R32NS1) and conalbumin, were encapsulated in separate liposomes. The mechanisms of presentation of unencapsulated and liposome-encapsulated R32NS1 and conalbumin to antigen-specific T-cell clones were investigated in in vitro antigen presentation assays using murine bone marrow-derived macrophages (BMs) as antigen-presenting cells. A much lower concentration of liposomal antigen than of unencapsulated antigen was required for T-cell proliferation. Liposome-encapsulated conalbumin required intracellular processing by BMs for antigen-specific T-cell proliferation, as determined by inhibition with chloroquine, NH_4Cl, leupeptin, brefeldin A, monensin, antimycin A, NaF, and cycloheximide and by treatment of BMs with glutaraldehyde. Liposome-encapsulated conalbumin therefore follows the classical intracellular antigen processing pathway described for protein antigens. Similarly, unencapsulated conalbumin also required intracellular processing for presentation to antigen-specific T cells. In contrast, both unencapsulated R32NS1 and liposome-encapsulated R32NS1 were presented to T cells by BMs without undergoing internalization and intracellular processing. These results suggest that the antigen itself is the major element that determines whether a requirement exists for intracellular processing of liposomal antigens by macrophages.

The mechanism of processing and presentation of liposomal antigens is not yet fully understood. It is known that soluble antigens that are rendered particulate by encapsulation are presented by macrophages to T cells and cause T-cell proliferation (8, 22). However, divergent theories on the exact mechanism of presentation of liposomal antigens have emerged. Harding et al. found that liposome-encapsulated hen egg lysozyme required processing in endocytic vesicles for activation of antigen-specific T cells (12). In this system, the liposomal antigen would therefore follow the classical intracellular antigen processing pathway described for soluble protein antigens in which exogenous protein antigens are internalized by antigen-presenting cells (APCs) and partially degraded to produce immunogenic peptides which bind to major histocompatibility complex (MHC) class II molecules. The MHC class II-peptide complex is then expressed on the surface of the APC, where it is recognized by the clonotypic T-cell receptor (13). Although this is the most widely accepted mechanism for presentation of exogenous soluble antigens, instances in which intracellular processing of antigens is not required have been reported (16, 17).

In contrast to the findings described above, other investigators have provided a different view of the mechanism of presentation of liposomal antigen. In studies using liposomes containing MHC class II molecules and a covalently attached protein antigen, Dal Monte and Szoka (8), Walden et al. (25), and Bakouche and Lachman (4, 5) have shown that cloned helper T cells and T-cell hybridomas can be stimulated in an antigen-specific MHC-restricted manner in the absence of APCs. Bakouche and Lachman (6) have further demonstrated that the requirement for intracellular processing for conalbumin can be bypassed by coupling conalbumin to the surface of liposomes containing MHC class II molecules and membrane interleukin 1.

In our previous in vitro studies dealing with the phagocytic interaction of liposomes with bone marrow-derived macrophages (BMs), we have demonstrated that liposome-encapsulated R32NS1 [L(R32NS1)] is localized both in vacuoles and in the cytoplasm of BMs (23) and that L(R32NS1)-pulsed BMs present the antigen to an $(NANP)_{40}$-specific murine T-cell clone (22). In the present study, we have addressed the question of whether internalization and intracellular processing of the antigens L(R32NS1) and liposome-encapsulated conalbumin [L(conalbumin)] are required for activation of antigen-specific T cells.

Conalbumin is a soluble protein antigen that requires processing for presentation to T cells (4). R32NS1 is a recombinant fusion protein that contains epitopes derived from the immunodominant region of the circumsporozoite protein of *Plasmodium falciparum*, whose processing and presentation characteristics are unknown. Our results demonstrate that under conditions in which intracellular processing mechanisms are directly inhibited, conalbumin and L(conalbumin) are not presented to a conalbumin-specific T-cell clone. In contrast, R32NS1 that is either unencapsulated or encapsulated in liposomes does not require internalization or processing within endocytic vesicles to activate T cells in an antigen-specific MHC-restricted manner. These data therefore suggest that the antigen itself is a major element in determining whether a requirement exists for intracellular processing and presentation of the antigen when it is encapsulated in liposomes. Our evidence supports the concept that presentation of liposomal antigens by macrophages can follow either of two alternative routes: intracellular processing followed by presentation, or cell surface presentation without intracellular processing.

MATERIALS AND METHODS

Mice. C57BL/10, B10.Br, and BALB/c mice were purchased from Jackson Laboratory, Bar Harbor, Maine. Mice were handled and housed according to the

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protocol approved by the small animal committee of the Walter Reed Army Institute of Research. Mice were given standard laboratory food and water ad libitum.

Reagents. Dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, and cholesterol were purchased from Avanti Polar Lipids, Inc., Alabaster, Ala. Glutaraldehyde, lysine, brefeldin A, leupeptin, cycloheximide, monensin, chloroquine, and antimycin A were purchased from Sigma Chemical Co., St. Louis, Mo. Sodium fluoride and NH_4Cl (AR grade) were purchased from Fisher Scientific Co., Fair Lawn, N.J. Recombinant murine gamma interferon was purchased from Amgen, Thousand Oaks, Calif.

Preparation of liposomes. Detailed procedures for the preparation of liposomes were described previously (3). Briefly, multilamellar liposomes, composed of dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, and cholesterol, in molar ratios of 1.8:0.2:1.5, were prepared by dispersion of lyophilized mixtures of lipids in Dulbecco's phosphate-buffered saline (PBS) containing or lacking R32NS1 or conalbumin. The liposomes were washed twice with 0.15 M NaCl to remove unencapsulated antigen, resuspended in buffer to give a final phospholipid concentration of 10 mM, and stored at 4°C until used. The amount of antigen encapsulated in liposomes was determined by a modified Lowry procedure (2, 3).

Antigens. R32NS1 (a kind gift from SmithKline Beecham Pharmaceuticals, King of Prussia, Pa.) is a recombinant protein containing 30 repeats of the tetrapeptide NANP interspersed with two repeats of the tetrapeptide NVDP derived from the immunodominant repeat region of the circumsporozoite protein of *P. falciparum*, fused to 81 amino acids of the nonstructural protein of influenza virus (NS1) (23, 28). Conalbumin type I from chicken egg white was purchased from Sigma. The superantigens staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB) were kind gifts from Jack Komisar (Walter Reed Army Institute of Research).

Antibodies. Hybridomas producing monoclonal antibodies against MHC class II molecules (anti-$I\text{-}A^{b,d}$ [TIB 120], anti-$I\text{-}A^{k}$ [TIB 93], and anti-$I\text{-}E^{k}$ [14.4.4S]) were obtained from the American Type Culture Collection (Rockville, Md.). Antibodies were purified from hybridoma culture supernatants by chromatography on protein A-Sepharose columns (Pharmacia Fine Chemicals, Piscataway, N.J.) and used at the indicated concentrations.

APCs. BMs were isolated and cultured as described previously (23). Marrows from femurs of 8- to 10-week-old mice were isolated, and cells were seeded in triplicate at a density of 5×10^4 cells per well in 96-well flat-bottom Costar tissue culture plates (Costar, Cambridge, Mass.) in macrophage growth medium (RPMI 1640 [Gibco Laboratories, Grand Island, N.Y.] containing 10% fetal bovine serum, 10% L-929-conditioned medium [7, 23], 8 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml). On day 9, macrophage cultures were supplemented with 10 U of murine gamma interferon per ml and used as APCs the next day.

T cells. The $(NANP)_{40}$-specific T-cell clone 11C6 ($H\text{-}2^{b}$ restricted), originally obtained from G. Corradin (University of Lausanne, Lausanne, Switzerland), was maintained in the laboratory as described previously (19). 11C6 cells (10^5) were stimulated with 0.16 μg of $Y(NANP)_3$ and irradiated syngeneic spleen cells (5×10^6) once every 3 weeks. Although the tyrosine residue is not present in the native circumsporozoite protein, it has been shown that the peptide $Y(NANP)_3$ gives the best proliferative response when used as an antigen in vitro (20). Conalbumin-specific T-cell clone D10.G4.1 (14) was kindly provided by R. Hodes (National Institutes of Health, Bethesda, Md.). This clone proliferates in response not only to conalbumin presented in the context of syngeneic $I\text{-}A^{k}$ but also to $I\text{-}A^{b}$ alloantigen. D10.G4.1 cells were stimulated every 7 to 10 days with 50 μg of conalbumin, irradiated syngeneic spleen cells, and rat spleen concanavalin A supernatant as a source of interleukin 2. Viable T cells were harvested by centrifugation over lympholyte M (Accurate Chemical & Scientific Corporation, Westbury, N.Y.). All cells were maintained in a humidified atmosphere at 37°C and 5% CO_2. T cells from C57BL/6 mice were purified by passage of splenic T cells over a nylon wool column (21).

Antigen presentation assay. BMs were used as APCs in T-cell proliferation assays because they represent a homogeneous cell population (1). The assay was performed as described earlier (22). Macrophage growth medium was replaced with RPMI 1640 (100 μl per well) before the addition of the antigen. BMs were pulsed for 90 min with different concentrations of R32NS1, conalbumin, liposome-encapsulated $Y(NANP)_3$ {$L[Y(NANP)_3]$}, L(R32NS1), or L(conalbumin) in RPMI 1640 in 100-μl aliquots. The medium was then aspirated, and the cells in each well were washed three times with 200 μl of RPMI 1640 maintained at 37°C to remove any unbound antigen. Macrophage cultures were exposed to 3,000-R irradiation from a cobalt source (Gammacell 220 cobalt 60 irradiator; Atomic Energy of Canada Ltd.). The medium was aspirated from the wells and replaced with T-cell growth medium [RPMI 1640 containing EHAA [1:1, vol/vol; Whittaker M. A. Bioproducts, Walkersville, Md.], 5% fetal bovine serum [HyClone Laboratories, Inc., Logan, Utah], 8 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 100 μM 2-mercaptoethanol). T-cell clones (10^4) were added to each well in a volume of 100 μl and cultured for 72 h. The cells were labeled in the final 16 h of the incubation with 1 μCi of [^{3}H]thymidine ([^{3}H]TdR; specific activity, 6.7 Ci/mM; NEN, Boston, Mass.) per well and subsequently harvested onto glass fiber filters and processed for scintillation spectrometry (LKB Betaplate). Data are expressed as the mean counts per minute of [^{3}H]TdR uptake ± standard deviation of triplicate cultures. Each experiment was repeated between three and six times.

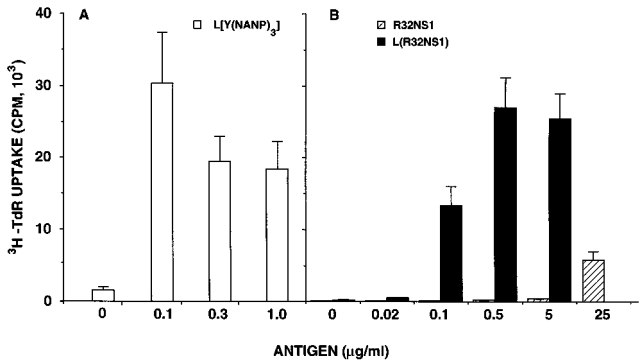

FIG. 1. Presentation of soluble and liposome-encapsulated R32NS1 and $L[Y(NANP)_3]$ by BMs to an antigen-specific T-cell clone. The $H\text{-}2^{b}$-restricted $(NANP)_{40}$-specific T-cell clone 11C6 (10^4 cells per well) was cultured for 72 h with irradiated autologous BMs (5×10^4 cells per well) previously pulsed for 90 min with the indicated concentrations of R32NS1, L(R32NS1), or $L[Y(NANP)_3]$. During the last 16 h of the culture, 1 μCi of [^{3}H]TdR was added. Cells were harvested and processed for scintillation spectrometry. Data shown from a representative experiment are expressed as the mean counts per minute of [^{3}H]TdR uptake ± standard deviation of triplicate cultures.

To test the effects of inhibitors on antigen processing and presentation, BMs were preincubated for 45 min with the inhibitors chloroquine (0 to 0.3 mM), NH_4Cl (0 to 10 mM), leupeptin (0 to 1 μg/ml), brefeldin A (0 to 10 μg/ml), monensin (0 to 5 μM), antimycin A (0 to 10 μg/ml), and NaF (0 to 10 mM) or for 4 h with cycloheximide (0 to 100 μM). R32NS1 (25 μg/ml), L(R32NS1) (0.5 μg/ml), conalbumin (250 μg/ml), or L(conalbumin) (5 μg/ml) was then added to the BM cultures for an additional 90 min of incubation. BM cultures were then treated as described above.

To test if L(R32NS1) behaves like a superantigen, BMs were pulsed with L(R32NS1) for 2 h and either irradiated as described above or first irradiated and then cultured with 10 μg of SEA or SEB per ml. In both cases, either 11C6 cells (10^4 per well) or purified T cells (3×10^5 per well) from C57BL/6 mice were added, and cultures were incubated for 72 h. Irradiated spleen cells (3×10^5) from C57BL/6 or B10.Br mice were also cultured for 72 h with either L(R32NS1), SEA, or SEB and 11C6 cells or purified syngeneic T cells. T-cell proliferation was measured as described above.

Glutaraldehyde fixation of BMs. The BMs were fixed with glutaraldehyde (grade II; 25% aqueous solution; Sigma), 0.05% (vol/vol) final concentration, for 45 s at room temperature and blocked with 0.2 M lysine in Hanks balanced salt solution (pH 7.4). The BMs were washed three times and pulsed with R32NS1, L(R32NS1), conalbumin, or L(conalbumin) for 90 min. The cells were then washed and irradiated, and T-cell clones were added. T-cell proliferation was measured as described above.

Sephadex G-50 column and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). R32NS1 (1.6 mg) was applied to a Sephadex G-50 column (20 by 1 cm) and eluted with Dulbecco's PBS (pH 7.2) (Gibco). Fractions of 0.44 ml were collected and analyzed by the Bio-Rad microassay, which was performed on microtiter plates. The absorbance was read in a Vmax Kinetic microplate reader (Molecular Devices, Palo Alto, Calif.), using a filter at 650 nm.

For SDS-PAGE analysis, approximately 10 μg of L(R32NS1) and an equal volume of empty liposomes were subjected to chloroform-methanol extractions to remove the lipids as described by Wessel and Flugge (27). The precipitates were air dried and solubilized in SDS sample buffer containing 2-mercaptoethanol. Samples were analyzed on an SDS–4 to 15% polyacrylamide (Bio-Rad) reducing minigel as described by Laemmli (15). The gels were stained with Coomassie blue or with silver, using the Bio-Rad silver staining kit.

RESULTS

Presentation of L(R32NS1) by BMs induces specific proliferation of T-cell clones. BMs were used as APCs to study the mechanism of presentation of L(R32NS1) to an $(NANP)_{40}$-specific T-cell clone, 11C6. This clone also recognized the peptide $Y(NANP)_3$ when encapsulated in liposomes (Fig. 1A). An L(R32NS1) concentration as low as 0.1 μg/ml caused T-cell proliferation, with enhanced activity at 0.5 μg/ml (Fig. 1B). Increasing the concentration of L(R32NS1) from 0.5 to 5 μg/ml did not cause any further enhancement of T-cell activa-

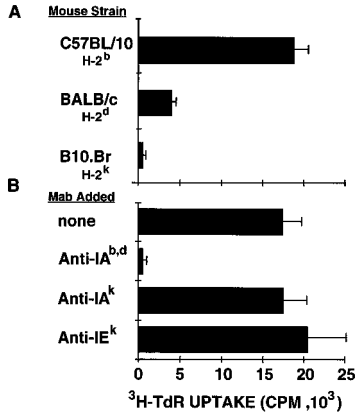

FIG. 2. Presentation of L(R32NS1) by BMs to 11C6 is class II MHC restricted. (A) BMs from different murine MHC haplotypes were pulsed with 0.5 μg of L(R32NS1) per ml for 90 min and were then washed and irradiated. (B) Purified monoclonal antibodies TIB 120 (anti-$I\text{-}A^{b,d}$), TIB 93 (anti-$I\text{-}A^{k}$), and 14.4.4S (anti-$I\text{-}E^{k}$) specific for haplotype-specific murine class II MHC molecules were added at 10 μg/ml to antigen-pulsed irradiated C57BL/10 BMs prior to the addition of 11C6 cells. The T-cell response was assayed as described in the legend to Fig. 1.

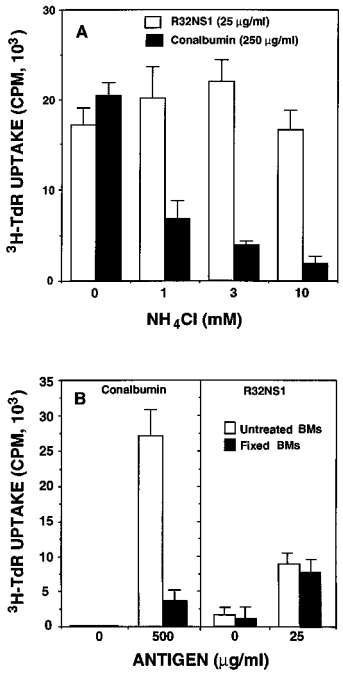

FIG. 3. Effects of NH_4Cl and glutaraldehyde fixation on presentation of R32NS1 and conalbumin. (A) BMs from C57BL/10 or B10.Br mice were preincubated with medium or different concentrations of NH_4Cl for 45 min. R32NS1 or conalbumin was then added for an additional 120 min (R32NS1) or 90 min (conalbumin) of incubation prior to washing, irradiation, and the addition of the T-cell clone 11C6 or D10.G4.1. (B) BMs from C57BL/10 and B10.Br mice were either untreated or fixed with 0.05% glutaraldehyde for 45 s, washed extensively, and pulsed with various concentrations of conalbumin or R32NS1 for 90 min. Cells were washed and irradiated, and the T-cell clone 11C6 or D10.G4.1 was then added. The T-cell responses were measured as described in the legend to Fig. 1. Data shown are from a representative experiment.

tion. In contrast, a 250-fold excess of unencapsulated R32NS1 compared with L(R32NS1) was required for T-cell proliferation [25 μg of R32NS1 per ml versus 0.1 μg of L(R32NS1) per ml] (Fig. 1B).

The optimum time for pulsing with the antigen was determined to be 90 min, using 0.5 or 1.0 μg of L(R32NS1) per ml (data not shown). Therefore, an L(R32NS1) concentration of 0.5 μg/ml and an antigen pulse time of 90 min were used in further experiments.

Presentation of L(R32NS1) to T cells is MHC restricted. To investigate whether the response of 11C6 T cells to L(R32NS1) was restricted by class II MHC molecules, BMs from different mouse strains, representing $H\text{-}2^{b}$, $H\text{-}2^{d}$, and $H\text{-}2^{k}$ haplotypes, were pulsed with 0.5 μg of L(R32NS1) per ml for 90 min before the addition of 11C6 cells. T-cell proliferation occurred only when L(R32NS1) was presented by syngeneic $I\text{-}A^{b}$ APC (Fig. 2A). In separate cultures, syngeneic BMs were incubated with monoclonal antibodies directed against the haplotype-specific class II MHC molecules to confirm the MHC restriction. The T-cell response was abrogated (Fig. 2B) by the addition of TIB 120 (anti-$I\text{-}A^{b,d}$), but not by the addition of TIB 93 (anti-$I\text{-}A^{k}$) or 14.4.4S (anti-$I\text{-}E^{k}$), thus supporting the conclusion that presentation of L(R32NS1) to 11C6 T cells is MHC restricted.

In addition to the MHC requirement, presentation of L(R32NS1) requires the presence of APCs. No proliferative response to either R32NS1 or L(R32NS1) was observed when antigen-specific T cells were incubated with R32NS1 or L(R32NS1) (data not shown). This finding indicated that in the absence of APCs, T cells did not present the antigen.

Ammonium chloride and glutaraldehyde fixation inhibits the presentation of conalbumin but not of R32NS1 to antigen-specific T cells. Conalbumin is a soluble protein antigen that requires intracellular processing for presentation to conalbumin-specific T cells. However, the processing and presentation requirements for R32NS1 have not been studied. To address this question, BMs from C57BL/10 and B10.Br mice were preincubated for 45 min with various concentrations of NH_4Cl. Conalbumin (250 μg/ml) or R32NS1 (25 μg/ml) was then added to the BM cultures for an additional 90 or 120 min of incubation, respectively. The optimum time for pulsing with R32NS1 was determined to be 120 min with 25 μg of antigen per ml (data not shown). NH_4Cl inhibited the processing of conalbumin but did not inhibit the processing of R32NS1 (Fig. 3A), thereby suggesting that R32NS1 behaves differently than conalbumin in that it does not require processing within acidic vesicles.

To further prove that intracellular processing of R32NS1 by BMs is not required for T-cell proliferation, BMs from C57BL/10 and B10.Br mice were prefixed with glutaraldehyde before being pulsed with the antigens. As shown in Fig. 3B, glutaraldehyde fixation of BMs from B10.Br mice completely inhibited the presentation of conalbumin to the conalbumin-specific T-cell clone. In contrast, fixation of BMs from C57BL/10 mice did not inhibit the presentation of R32NS1 to 11C6 cells (Fig. 3B). These results support the conclusion that in contrast to conalbumin, presentation of R32NS1 to 11C6 cells does not require internalization or intracellular processing by BMs.

Presentation of L(R32NS1) but not L(conalbumin) by BMs to T cells is unaffected by inhibitors of intracellular processing and transport of proteins. Using BMs as APCs, we previously demonstrated that L(R32NS1) is efficiently phagocytosed by BMs (23) and presented to 11C6 T cells (22). In the experiments described below, we examined (i) whether internalization and intracellular processing of L(R32NS1) is required for T-cell activation, since presentation of R32NS1 is unaffected by treatment with NH_4Cl and glutaraldehyde fixation, and (ii) whether encapsulating conalbumin in liposomes would change the processing requirements for conalbumin-specific T-cell ac-

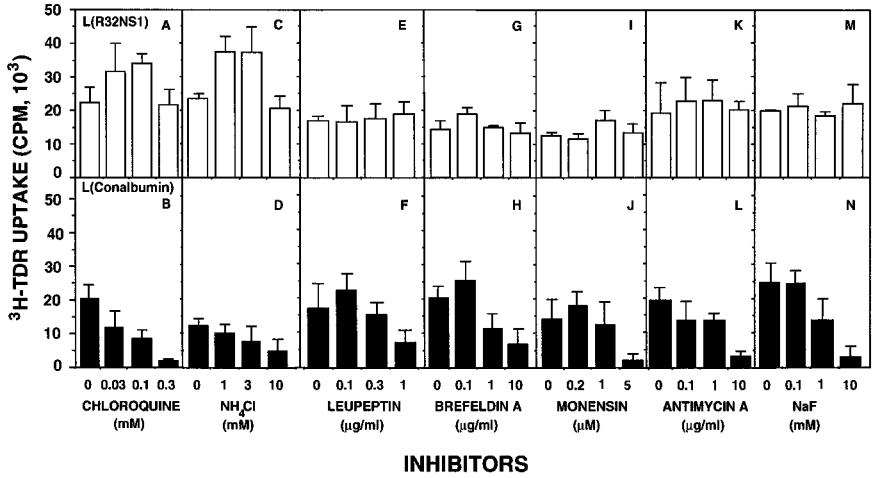

FIG. 4. Effects of inhibitors on the processing and presentation of L(R32NS1) and L(conalbumin). C57BL/10 (*H-2*^{b}) (top panels) or B10.Br (*H-2*^{k}) (bottom panels) BMs were preincubated with medium or with different concentrations of chloroquine (A and B), NH_4Cl, (C and D), leupeptin (E and F), brefeldin A (G and H), monensin (I and J), antimycin A (K and L), or NaF (M and N) in separate experiments for 45 min. L(R32NS1) (0.5 μg/ml; top panels) or L(conalbumin) (5 μg/ml; bottom panels) was then added for an additional 90 min of incubation prior to washing, irradiation, and the addition of the T-cell clone 11C6 or D10.G4.1. The T-cell responses were assayed as described in the legend to Fig. 1. Data shown are from a representative experiment. The background [^{3}H]TdR uptake ranged from 280 to 2,600 cpm.

tivation. Inhibitors that block either internalization or intracellular processing of proteins were added separately to BMs during pulsing with the antigens.

BMs from C57BL/10 and B10.Br mice were preincubated for 45 min with appropriate inhibitors before the addition of L(R32NS1) (Fig. 4, top panels) or L(conalbumin) (Fig. 4, bottom panels). Lysosomotrophic agents (chloroquine and NH_4Cl) inhibited the processing of L(conalbumin) (Fig. 4B and D) but did not inhibit the processing of L(R32NS1) (Fig. 4A and C). These results suggested that L(conalbumin) but not L(R32NS1) was processed within an endosomal-lysosomal compartment. This latter conclusion was further supported by the observation that leupeptin, a thiol protease inhibitor, did not affect the processing and presentation of L(R32NS1) (Fig. 4E) but inhibited the presentation of L(conalbumin) to D10.G4.1 cells (Fig. 4F).

Although L(R32NS1) was not degraded into peptides in acidic vesicles, the antigen might still have been internalized to associate with either newly synthesized or recycling MHC class II molecules before being exported as a complex to the cell surface. To examine whether newly synthesized class II molecules were involved, the effects of brefeldin A and monensin on presentation of L(R32NS1) to T cells were tested. Brefeldin A inhibits the transport of newly synthesized class II molecules from the endoplasmic reticulum without affecting the recycling class II population, whereas monensin inhibits intravesicular transport. Both brefeldin A and monensin inhibited the presentation of L(conalbumin) to D10.G4.1 cells (Fig. 4H and J) but did not inhibit the presentation of L(R32NS1) to 11C6 cells (Fig. 4G and I). Furthermore, cycloheximide did not inhibit the presentation of L(R32NS1) to T cells (Table 1). These results support the conclusion that presentation of L(conalbumin) but not L(R32NS1) depends upon newly synthesized or recycling class II MHC molecules.

To further confirm the observation that internalization of L(R32NS1) was not necessary for T-cell proliferation, the effects of two phagocytic inhibitors, antimycin A and sodium fluoride, were tested. Both antimycin A, an inhibitor of oxidative phosphorylation, and sodium fluoride, an inhibitor of glycolysis, interfered with the presentation of L(conalbumin) (Fig. 4L and N), but neither inhibitor affected L(R32NS1) presentation to 11C6 cells (Fig. 4K and M). These data therefore further demonstrate that L(conalbumin) requires internalization and intracellular processing by BMs in order to cause proliferation of D10.G4.1 cells, while phagocytosis of L(R32NS1) by BMs is not required for proliferation of 11C6 cells.

Glutaraldehyde-fixed BMs present L(R32NS1) to T cells. To further prove that intracellular processing of L(R32NS1) by BMs is not required for T-cell proliferation, BMs from C57BL/10 and B10.Br mice were prefixed with glutaraldehyde before being pulsed with the antigens. As shown in Fig. 5A, glutaraldehyde fixation of BMs from B10.Br mice completely inhibited the presentation of L(conalbumin) to the conalbumin-specific T-cell clone. In contrast, as seen in the case of R32NS1, fixation of BMs from C57BL/10 mice did not inhibit the presentation of L(R32NS1) to 11C6 cells (Fig. 5B). These results support the conclusion that in contrast to L(conalbumin), presentation of L(R32NS1) to 11C6 cells does not require internalization or intracellular processing by BMs.

TABLE 1. Effect of cycloheximide on the presentation of L(R32NS1) to T cells[a]

Antigen concn (μg/ml)	[^{3}H]TdR uptake (10^3 cpm) at cycloheximide concn of:			
	0 μM	10 μM	30 μM	100 μM
L(R32NS1)				
0.0	1.70	ND	1.51	1.65
0.5	17.18	20.48	28.74	16.78
1.0	18.44	21.24	22.80	20.32
L(conalbumin)				
0.0	0.21	0.62	0.78	0.45
5.0	12.18	3.98	1.28	0.54

[a] C57BL/10 or B10.Br BMs were preincubated with different concentrations of cycloheximide for 4 h. After the addition of L(R32NS1) or L(conalbumin), the cultures were incubated for an additional 90 min. The responses of the T-cell clones 11C6 and D10.G4.1 were assayed as described in the legend to Fig. 1. Data are expressed as the means of triplicate cultures. ND, not determined.

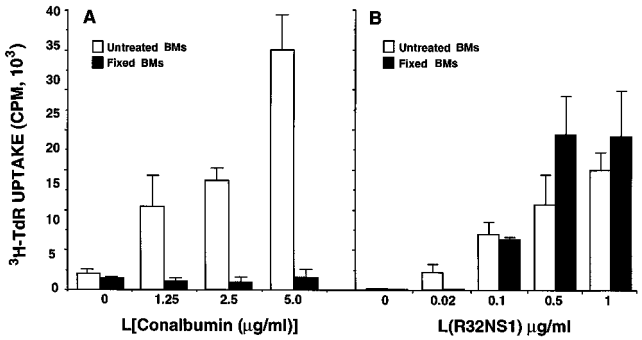

FIG. 5. Effect of glutaraldehyde fixation on presentation of L(R32NS1) and L(conalbumin). BMs from C57BL/10 and B10.Br mice were either untreated or fixed with 0.05% glutaraldehyde for 45 s, washed extensively, and pulsed with various concentrations of L(conalbumin) (A) or L(R32NS1) (B) for 90 min. Cells were washed and irradiated, and the T-cell clone 11C6 or D10.G4.1 was then added. The T-cell responses were measured as described in the legend to Fig. 1. The data represent at least three experiments.

Lack of processing of R32NS1 is not due to the presence of degraded fragments. To rule out the possibility that the lack of processing requirement for R32NS1 or L(R32NS1) is due to the presence of contaminating peptide fragments of R32NS1 in the preparations used, R32NS1 was chromatographed on a Sephadex G-50 column. As shown in Fig. 6A, R32NS1 eluted as a single peak. This result indicated that the protein preparation did not contain any peptide fragments. To further confirm this result, R32NS1 and L(R32NS1) were subjected to SDS-PAGE. Samples were analyzed on a 4 to 15% polyacrylamide gradient to visualize any low-molecular-weight peptides. Both R32NS1 and L(R32NS1) migrated as a single band, as evidenced by Coomassie blue (Fig. 6B) and silver (Fig. 6C) staining, thereby ruling out the presence of any peptide fragments in the preparations.

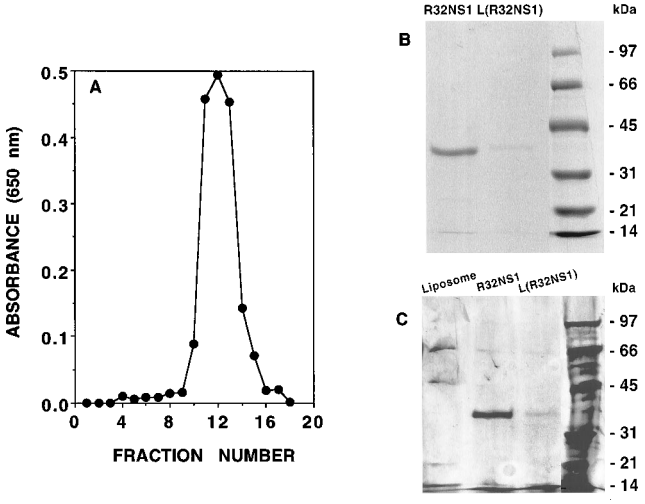

FIG. 6. Sephadex G-50 column chromatography and SDS-PAGE analysis. (A) R32NS1 (1.6 mg) was chromatographed on a Sephadex G-50 column (20 by 1 cm). The protein was eluted with PBS. Each fraction was assayed for protein by the Bio-Rad microassay. The assay was performed on microtiter plates and read in a plate reader at 650 nm. R32NS1 (6 μg), empty liposomes, and L(R32NS1) were examined on an SDS–4 to 15% gradient minigel by PAGE (see Materials and Methods for details). (B) Coomassie blue staining. (C) Silver staining. Molecular mass markers are shown on the right. The gel was photographed, and the computer-generated image was produced with the negative scanned in by using the Adobe Photoshop software.

L(R32NS1) is not a superantigen. To test the possibility that L(R32NS1) behaved like a superantigen, BMs or irradiated spleen cells from C57BL/6 or B10.Br mice were cultured with L(R32NS1) or a known superantigen, SEA or SEB, and proliferation of purified syngeneic T cells was measured. As shown in Table 2, and as has been well documented, T-cell proliferation in response to superantigens does not require antigen specificity. In contrast, only antigen-specific T cells (11C6) proliferate in response to L(R32NS1) presented by either BMs or irradiated splenic cells. Also, as shown in Fig. 2, presentation of L(R32NS1) to 11C6 is IA^b restricted, thus establishing that L(R32NS1) does not act like a superantigen.

DISCUSSION

In this study, the mechanisms of presentation of two liposome-encapsulated antigens, R32NS1 and conalbumin, by BMs to antigen-specific T-cell clones were investigated. Our in vitro antigen presentation system, consisting of BMs as APCs, has the advantage of comprising a homogeneous cell population (1) that can internalize particulate antigens by phagocytosis (23). Cellular homogeneity is especially important in studies that are examining the mechanism of antigen presentation because of the need to rule out contributions by other APCs that might be present.

With this system, we demonstrated that L(conalbumin) followed the classical pathway for processing of soluble antigens, in that internalization and intracellular processing of the liposomal antigen within endocytic vesicles were required for activation of conalbumin-specific T cells. In contrast, presentation of L(R32NS1) to an antigen-specific T-cell clone was unaffected by treatment of BMs with inhibitors that blocked either internalization or intracellular processing of proteins. Furthermore, L(R32NS1) was presented equally well by untreated or glutaraldehyde-fixed BMs. From these results, we conclude that L(R32NS1) does not require internalization or processing within intracellular acidic compartments to activate T cells in an antigen-specific MHC-restricted manner.

It is well known that certain hydrophobic molecules, including liposomes, can gain entry into the cytoplasm of cells in the absence of endocytosis through the mechanism of fusion of hydrophobic regions of lipids or other hydrophobic molecules with hydrophobic regions of cell membranes. In support of this finding, it has been demonstrated that fluorescence-labeled liposomal lipids can enter the cytoplasm of glutaraldehyde-fixed cells (18). Therefore, in this study, a number of inhibitors that block intracellular processing of proteins were used to demonstrate that any liposomal R32NS1 constituents that may have entered the cells in this manner were not necessary for processing of R32NS1 for presentation to antigen-specific T cells.

Although liposomal conalbumin and conalbumin require intracellular processing whereas liposomal R32NS1 does not, we found that presentation of unencapsulated R32NS1 was also unaffected by treatment with NH_4Cl or glutaraldehyde fixation. We conclude, therefore, that the type of antigen used for encapsulation in liposomes can influence the requirement for intracellular processing of liposomal antigen by macrophages before presentation to T cells. Liposome-encapsulated antigens theoretically can follow either of two alternative mechanisms: intracellular processing of the liposomal antigen before presentation to T cells or cell surface presentation in the absence of intracellular processing. Furthermore, encapsulation of antigens in liposomes, although rendering the antigens particulate and liable to be phagocytosed by macrophages (23),

TABLE 2. Responses of antigen-specific and naive T cells to L(R32NS1) presented by different APCs[a]

Expt	APCs	T cells	Antigen	Proliferative response (10^3 cpm)	Stimulation index (antigen cpm/ background cpm)
1	BMs	11C6	None	1.62	
			L(R32NS1)	28.03	17.30
			SEA	0.30	0.18
			SEB	4.02	2.48
	Irradiated spleen cells	11C6	None	0.20	
			L(R32NS1)	12.94	64.70
	BMs	Purified C57BL/6 T cells	None	0.41	
			L(R32NS1)	0.59	1.44
			SEA	1.32	3.22
			SEB	4.79	11.68
	Irradiated spleen cells	Purified C57BL/6 T cells	None	1.84	
			L(R32NS1)	2.18	1.18
			SEA	481.16	261.50
			SEB	114.15	62.04
2	Irradiated spleen cells	Purified B10.Br T cells	None	0.11	
			L(R32NS1)	0.08	0.73
			SEA	5.88	53.45
			SEB	2.60	23.64

[a] Irradiated spleen cells from C57BL/6 or B10.Br mice were cultured with 10 μg of SEA or SEB per ml or with 0.5 μg of L(R32NS1) per ml in the presence of 11C6 cells (10^4 per well), purified C57BL/6 T cells (3×10^5 per well), or purified B10.Br T cells (10^4 per well) for 72 h. BMs from C57BL/6 mice either were irradiated and cultured with 10 μg of SEA or SEB per ml as described above or were pulsed with 0.5 μg of L(R32NS1) per ml for 90 min and then irradiated before the addition of either 11C6 cells or purified naive syngeneic T cells. The responses of the T cells were assayed as described in the legend to Fig. 1.

does not necessarily lead to a requirement for intracellular processing as the sole mechanism for presentation to T cells.

With respect to the controversial issues described earlier (see the introduction), our results with L(conalbumin) are in agreement with those of Harding et al. (12), who showed that liposomal hen egg lysozyme requires intracellular processing before presentation to T-cell hybridomas. However, Bakouche and Lachman (6) showed that conalbumin when coupled to the surface of liposomes containing class II molecules and membrane interleukin 1 could be presented to conalbumin-specific T-cell clones in the absence of intracellular processing. Clearly, covalent coupling of conalbumin (6) rather than encapsulation in liposomes (this study) could have affected the requirement for antigen processing.

In general, protein antigens require intracellular processing in order to activate T cells. Protein antigens that do not require processing are rare; for example, T-cell responses to microbial superantigens do not require processing (10), human fibrinogen specifically activates T-cell hybridomas without being processed (16), and lactose dehydrogenase-specific T cells are activated by lactose dehydrogenase incorporated into liposomal vesicles containing the appropriate class II molecules in the absence of APCs (24). Planar membranes containing purified Ia molecules as the only protein also are able to present an immunogenic peptide to T-cell hybridomas (26).

Most of the antigens that have been previously used in studying antigen processing have consisted of well-characterized soluble globular proteins. On the other hand, presentation of parasitic antigens and, in particular, particulate antigens has so far not received much attention. In this study, we found that the processing requirements of liposomal conalbumin, representing a soluble globular protein rendered particulate by encapsulation in liposomes, were different from those of liposomal R32NS1, which represents a parasitic protein. Our results also demonstrate that presentation of R32NS1, an antigen that does not require intracellular processing by itself, is not forced into an exclusive intracellular processing route for presentation to T cells by encapsulation in liposomes. It is possible that antigens, such as R32NS1, which contain repeat motifs behave fundamentally differently than conventional protein antigens lacking repeat motifs. In support of this view, it has recently been demonstrated that *Plasmodium berghei* sporozoites, which also contain a repeat motif in the circumsporozoite protein, do not require intracellular processing by APCs to activate antigen-specific T cells (17).

The question arises as to how presentation could have occurred in the absence of internalization of L(R32NS1) to generate immunogenic peptides. It should be pointed out that the absence of a requirement for intracellular processing of L(R32NS1) suggests that this antigen may act as if it were a superantigen. However, the observation that antigen-specific but not nonspecific T cells respond to L(R32NS1) in an MHC-restricted fashion rules out this possibility.

R32NS1 is an extremely hydrophobic protein and is at least partially inserted in the hydrophobic lipid bilayer of the liposome. Although we have no data yet that address the detailed molecular mechanism of presentation of R32NS1 or L(R32NS1) directly at the cell surface, one could speculate that liposomal R32NS1 had fused with the cell membrane, thereby orienting the antigen for binding to the class II molecule, or that R32NS1 could be partially unfolded and parts of the molecule could be available for binding to the MHC class II complex on the cell surface. The possibility that the interaction of R32NS1 with the MHC class II complex has low affinity is suggested by the requirement for a much higher amount of unencapsulated antigen than of encapsulated R32NS1 for T-cell activation (Fig. 1B). Encapsulation of R32NS1 in liposomes presents a broader and more concentrated surface area of antigen for contact with the cell surface, thereby increasing the efficiency of antigen presentation by BMs and effectively decreasing the concentration of antigen required for the T-cell activation. Our data are consistent with several previous studies that showed that proteins, when encapsulated or conjugated to liposomes, can cause enhanced T-cell activation (8, 9).

It is unlikely that any of the effects seen in this study were due to unencapsulated antigen having been released from the liposomes, since R32NS1 is tightly associated with the lipo-

somes. This is evidenced by the fact that drastic treatment with sodium deoxycholate is required for measurement of the residual amount of antigen that remains with lysed liposomes (3). Liposomes identical to these were used in a vaccine trial in humans (11), and little or no leakage was encountered over at least 42 months of storage (unpublished data). It is also possible that liposomal R32NS1 could have been degraded by proteases present in the fetal calf serum. To eliminate any involvement of proteases present in the culture medium, BMs were always pulsed with L(R32NS1) in the absence of fetal calf serum. Another possibility is that the L(R32NS1) preparation contained contaminating peptide fragments of R32NS1 and that these fragments were actually being presented by the fixed BMs. That this was not the case was evidenced by the fact that R32NS1 eluted as a single peak when chromatographed on a Sephadex G-50 column. Furthermore, PAGE analysis of unencapsulated R32NS1 and L(R32NS1) showed a single band, thereby ruling out any significant proteolysis of the antigen.

Previous studies have suggested that the mechanism by which liposomes exert potent activity as vehicles for vaccines is through internalization of liposomes by macrophages that serve as APCs. The present work suggests the additional mechanism that the presence of L(R32NS1) on the cell surface may also facilitate T-cell stimulation through a wide repertoire of APCs, such as B cells or dendritic cells, that are not capable of phagocytosis. The capability for surface presentation by APCs might serve to broaden the utility of liposomes for inducing antibody and cellular responses with certain liposomal antigens.

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