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Antigen Presentation by Liposomes Bearing Class II MHC and Membrane IL-1

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Liposomes containing membrane IL-1, Ia^k, and the antigen conalbumin were evaluated as "synthetic antigen presenting cells." The role of these three molecules in macrophage-T cell interaction was studied by testing their ability to induce the proliferation of a T-cell clone specific to conalbumin (the D10 cell line) or immune spleen cells sensitized three times *in vivo* with conalbumin. In the latter case, splenic macrophages were eliminated by adherence and a lysomotropic agent. The antigen conalbumin was presented on the surface of the liposomes as native undigested protein. When the liposomes presented native conalbumin, Ia^k, and membrane IL-1, significant proliferation occurred, but if the liposomes lacked membrane IL-1, the proliferation of the T-cell clone and the spleen cells reached only about 60 percent of the previous signal. Native conalbumin and class II antigen alone were required for T-cell activation, while membrane IL-1 only amplified the response. When the liposomes were made with only Ia^k and membrane IL-1, lacking conalbumin, there was no proliferation of antigen-specific target cells. These results indicated that in this synthetic system, membrane IL-1 increases the magnitude of the response but is not essential for the proliferative response of antigen-specific T cells.

INTRODUCTION

Artificial vesicles, such as liposomes, have been widely used in a variety of functional membrane systems [1] to increase the immunogenic properties of a soluble antigen required for the development of effective vaccines [2] or as a tool to explore the hapten-carrier phenomenon [3]. It has already been demonstrated that liposomes reconstituted with a variety of cell surface antigens can replace intact viable tumor cells in the induction of specific allogeneic and xenogeneic cytotoxic T lymphocytes *in vitro* [4,5]. In addition, appropriately designed liposomes can replace intact viable tumor cells in the induction of high levels of antibody directed against a syngeneic tumor antigen [6]. Thus, the association of complex cell-derived antigens with liposomes appears to enhance immune reactivity in a way similar to that described originally for defined haptens by Kinsky and Nicolotti [7].

The mechanisms by which the microenvironment and architecture of an antigen can regulate its ability to stimulate T cells are not yet well understood. The insertion into model membranes of antigens alone or in association with other molecules such as class II major histocompatibility complex (MHC) antigens and/or membrane interleukin (IL-1) and the use of these liposomes as functional antigens, immunogens, or artificial macrophages could enable us to understand better the molecular requirements for the

Abbreviations: APC: antigen presenting cell DTP: dithiopropionate FCS: fetal calf serum HBSS: Hank's buffered saline solution IL-1: interleukin 1 LPS: lipopolysaccharide MEM: minimum essential medium MHC: major histocompatability complex PBS: phosphate buffered saline TdR: thymidine

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induction of the immune response as well as for T-cell activation. Using artificial vesicles, Walden et al. [8] studied the minimal requirements of T-cell activation using an antigen presenting cell (APC)-cytokine-independent stimulus. For this purpose, they described the production of synthetic lipid vesicles with inserted class II MHC molecules and a protein antigen coupled covalently to the lipid to re-create their supramolecular organization. These vesicles were shown to stimulate cloned helper T cells and T-cell hybridomas in an antigen-specific MHC-restricted manner in the absence of APC [8]. In light of these observations, we constructed synthetic APC, using liposomes presenting on their surface native conalbumin, Ia class II MHC antigen, and membrane IL-1 in an attempt to determine the requirements for T-cell activation and the role of membrane Il-1 in macrophage-T-cell interaction.

MATERIALS AND METHODS

Mice

C_3H, C57BL/6, and DBA mice were purchased from the Animal Production Area, NCI-Frederick Cancer Research Facility (Frederick, MD) and were pathogen-free. The use of the animals, which were housed in The Smith Research Building facility, was approved by the Institution's Animal Care and Use Committee.

Chemicals and Biologicals

All the media used were endotoxin-free (detection limit <0.125 ng/ml) as determined by the limulus amebocyte lysate assay (Associates of Cape Cod, Inc., Woods Hole, MA). Lipopolysaccharide (LPS) (*Escherichia coli* 026:66), cholesterol, dithiothreitol (DTT), and conalbumin were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Thymidine (TdR) was purchased from New England Nuclear (Boston, MA). Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Avanti Polar Lipids (Birmingham, AL). N-Hydroxysuccinimidyl 3-(2-pyridyldithio) propionate (SPDP) was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Monoclonal antibodies directed against MHC antigens (I-a^k, I-a^b, I-a^d) were purchased from Litton Bionetics (Charleston, SC). Minimum Essential Medium (MEM)-Vitamins (100 X) were purchased from Mediatech (Washington, DC).

Collection of Peritoneal Mouse Macrophages

Thioglycollate-stimulated peritoneal macrophages were collected by peritoneal lavage from mice given 1.5-ml intraperitoneal injections of thioglycollate broth (Baltimore Biological Laboratories, Cockeysville, MD) five days before harvest [9]. The peritoneal exudate macrophages were washed three times (250 *g* for ten minutes) in medium and plated for 90 minutes on petri dishes in serum-free MEM (2 percent MEM-vitamins, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin). After 90 minutes, the non-adherent cells were removed by extensive washing and the remaining adherent cells were refed with MEM plus 10 percent fetal calf serum (FCS) with or without macrophage activators. These procedures routinely yield homogeneous preparations (>95 percent) of phagocytic macrophages, as determined by functional and morphological criteria [9].

Collection and Activation of Spleen Cells

Mice were sacrificed by cervical dislocation and the spleen removed and placed in a petri dish with Hank's buffered saline solution (HBSS) free of Ca^{2+}/Mg^{2+}. The spleens were minced in the same buffer and the splenocytes collected after three washes (1,500 rpm for ten minutes). LPS-stimulated spleen cells—lymphoblasts (LPS blasts)—were generated by a 64- to 84-hour culture of spleen cells in the following medium: RPMI 1640 supplemented with 2 mg/ml glucose, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, 5 mM HEPES, 1 mM sodium pyruvate, and 10 percent fetal calf serum with 50 μg/ml LPS.

Purification of the Class II MHC Antigens

LPS blasts have been shown to have normal levels of class I MHC antigen, but three to five times higher levels of class II antigens [10]. These cells were the source of I-A (C_3H for I-a^k, C57 BL/6 for I-a^b, and DBA for I-a^d). The plasma membranes were isolated as described [11]. Briefly, 5×10^{10} spleen cells were disrupted by a cell-rupturing pump, and the plasma membranes were purified using a 41 percent sucrose and a Percoll (Pharmacia) gradient under alkaline conditions [11]. Enzyme markers such as 5′ nucleotidase demonstrated that the plasma membranes were over 97 percent pure [11]. The membrane proteins were solubilized by a detergent treatment using 9 mM CHAPS (Pierce, Rockford, IL) and dialyzed against Dulbecco's phosphate buffered saline (PBS). A glycoprotein pool was obtained by chromatography on lentil lectin-4 B (Pharmacia) as desribed by Watts et al. [12]. The glycoproteins were eluted with 10 percent a-methyl-D-mannoside in 0.5 percent (weight per volume) sodium deoxycholate in 0.01 M Tris/0.14 M NaCl/0.02 percent NaN_3 pH 8.3 ($NaCl/NaN_3$). About 32 mg of glycoproteins were obtained from 5×10^{10} cells. Further purification was performed by affinity chromatography according to the procedure described by Turkewitz et al. [10] and by Watts et al. [12].

Monoclonal class II-specific antibodies used for the affinity columns were coupled to sepharose 4B by the cyanogen bromide method (>90 percent coupling efficiency). The glycoprotein pool was applied to the affinity column, which was then washed in 0.5 percent deoxycholate with 15 mM triethanolamine (pH 8). This process was used as a first step to remove the nonspecific contaminating proteins. The column was then washed with 20 volumes of 0.5 percent (weight per volume) deoxycholate in Tris/NaCl/NaN_3 and then with ten volumes of 30 mM octylglucoside in Tris/NaCl/NaN_3. The class II antigen was eluted with 2 M ammonium thiocyanate in octylglucoside/Tris/NaCl/NaN_3. Prior to its reconstitution, Ia was dialyzed against 0.5 percent sodium deoxycholate in Tris/NaCl/NaN_3. Sometimes dilute fractions were further concentrated on a 0.5-ml lentil lectin column before their reconstitution in liposomes.

Purification of Membrane IL-1

Peritoneal mouse macrophages were activated by 10 μg/ml of LPS for 16 hours and then the cells were disrupted by a cell-rupturing pump [11]. The plasma membranes were purified using a 41 percent sucrose and Percoll gradient under alkaline conditions [11]. The plasma membranes (over 97 percent purity) were suspended in 2 ml of 9 mM CHAPS and kept on ice for 30 minutes. The cell extract was dialyzed at 4°C for two days against DPBS and applied to a lentil lectin-sepharose 4B column to remove the membrane glycoproteins. The eluted solution was then applied on a monoclonal class

II-specific antibody column to remove the remaining class II molecules. This eluted solution was then fractionated by gel filtration chromatography using sephadex G50, and fractions containing IL-1 activity were identified, using the murine D10 assay (see below).

Construction of the Synthetic APC

Cholesterol was recrystallized three times from ethanol. Dipalmitoyl L-phosphatidylethanolamine 3-(2-pryidyldithio) propionate (DPPE-DTP) was prepared as follows: DPPE (10 μmol) was mixed in chloroform: methanol (9:1, 700 μl). SPDP (Pharmacia) (12 μmol in 300 μl of methanol), and then 20 μmol triethylamine were added. After a two-hour incubation at room temperature with stirring, the organic phase was washed with 2 ml of phosphate buffer (0.1 M, NaCl, pH 7.4) and then twice with water. The organic phase was dried under nitrogen and then lyophilized. The product was redissolved in chloroform: methanol (9:1). DPPC (13.9 μmol), cholesterol (5.6 μmol), and DPPE-DTP (0.7 μmol) were mixed and the organic solvent was first evaporated under nitrogen and then lyophilized for two hours. To the lipid film was added 3 ml L-buffer (0.01 M HEPES and 0.145 M NaCl, pH 7.45). Once lipids were suspended by vortexing, the liposomes formed.

Native conalbumin antigen was modified by SPDP as described by Barbet et al. [13]. Prior to SPDP modification, conalbumin (760 μg/ml) was transferred to phosphate buffer (0.1 M, pH 7.5, 0.1 M NaCl) and incubated with SPDP (20 mol of SPDP/mol of protein) for 30 minutes at room temperature to obtain dithiopropionate (DTP)-conalbumin. The DTP-conalbumin was activated by conversion of DTP groups to free thiol groups as described by Barbet et al. [13]. Briefly the DTP-conalbumin was incubated with DTT (50 mM final concentration) for 20 minutes at room temperature. Free thiol-bearing conalbumin, referred to as conalbumin-SH, was immediately coupled to liposomes: conalbumin-SH was added to liposomes containing DPPE-DTP and incubated at room temperature for 24 hours, a sufficient period for the reaction to come to completion [13]. Protein-bearing liposomes could be separated from uncoupled protein by centrifugation (one hour, 48,000 *g*) or by gel filtration on a small sepharose 4B column (5 × 1.5 cm) [14]. The protein-bearing liposomes were then lyophilized and resuspended in phosphate buffer containing both the IL-1 activity and class II MHC antigen. The foreign protein antigens used were then covalently linked to the lipids and both membrane IL-1 and class II MHC molecules inserted into the lipid bilayers by their hydrophobic properties.

D10 G4.1 T Cell Clone Assay for IL-1

The D10 IL-1-dependent T-cell clone was kindly provided by Dr. Charles Janeway and maintained in culture as previously described [11]. IL-1 activity was assayed by adding 1×10^4 D10 cells/well in 200 μl medium containing 10 percent FCS, 2.5 percent sodium pyruvate, 5×10^{-5} 2-mercaptoethanol, and 2.5 μg/ml concanavalin A. D10 cell proliferation was assessed by measuring [^{3}H]TdR incorporation during the final 24 hours of a 72-hour incubation at 37°C. Samples were assayed in twofold serial dilutions, and the activity determined as the reciprocal of the dilution, giving 50 percent maximal incorporation. The amount of IL-1 giving 50 percent maximal incorporation was defined as 1 unit/ml.

D10 Proliferation Using Synthetic APC

The D10 murine T-cell clone is IL-1-dependent and conalbumin antigen-specific; the murine T-cell line can proliferate in response not only to conalbumin presented in the context of syngeneic I-a^k (C_3H-spleen macrophages) but also to I-a^b alloantigen and to the membrane and soluble forms of IL-1 when co-cultured with concanavalin A [11]. Synthetic APC macrophages were built using liposomes bearing the different selected molecules and adjusted to 500 nmoles of lipid per milliliter of culture medium. The D10 cells were used no earlier than 21 days after exposure to the feeder cells (mouse C_3H spleen cells) and the conalbumin antigen. The D10 cells were centrifuged at 500 *g* for ten minutes and plated at 1×10^4 cells/well in 200 μl final D10 assay medium containing the synthetic macrophages. Antigen presentation was assayed by measuring D10 proliferation in response to the synthetic APC built as required and by measuring [^{3}H]TdR incorporation (0.2 μCi) overnight during the last 16 to 24 hours of a 72-hour incubation as described [15].

Immune Spleen Cell Proliferation

Mice were immunized three times using conalbumin-liposomes (liposomes bearing conalbumin antigen) and sacrificed by cervical dislocation. The spleens, harvested on the fourth week after the third immunization, were dissociated in HBSS without Ca^{2+}/Mg^{2+}. After three washes in this buffer, the splenic macrophages were removed by a leucin-O-methylester (LeuOMe) treatment (5 mM in RPMI 1640 serum-free medium) for 40 minutes at 22°C as described [16] and by adherence. The spleen cells were further washed (1,500 rpm for ten minutes), and 2×10^5 cells in 0.1 ml of RPMI 1640 medium supplemented with 5 mM glutamine, 10 mM HEPES buffer, 5×10^{-5} M 2-mercaptoethanol, 10 percent FCS were evenly distributed in a 96-well plate. In the first experiment, serial dilutions of synthetic APC were added to determine the right protein concentrations, and in the later experiments appropriate dilutions of synthetic APC (100 μl in triplicate) were added to each well. The plates were incubated at 37°C in a humidified atmosphere supplemented with 5 percent CO_2 for five days. On the last day, 0.2 μCi of [^{3}H]TdR was added to the culture (overnight labeling), and the cells were harvested on an automatic cell harvester.

RESULTS

D10 Cell Proliferation: Requirements for Native Antigen

To determine the minimal requirements for T-cell activation, synthetic APCs were prepared, using DPPC/cholesterol/DPPE liposomes bearing the class II MHC molecules (Ia) and membrane IL-1 hydrophobically bound to the lipids and the native conalbumin antigen covalently linked to the phospholipids (Fig. 1). These synthetic APCs were then tested for their ability to induce the proliferation of the conalbumin-specific D10 T-cell clone. As shown in Table 1, when synthetic APCs were made with conalbumin, I-a^k, and membrane IL-1, D10 proliferation was maximal and optimal (100 percent = 125,000 cpm). The proliferation signal of the D10 cells decreased to 62 percent when the IL-1 was removed. When the lipsomes bore I-a^k and membrane IL-1 only, the proliferation was weak (5 percent), but the construction of synthetic APC with conalbumin antigen, I-a^b, and membrane IL-1 produced a D10 proliferation signal 56 percent of maximal. In the absence of membrane IL-1, the proliferation signal dropped down to 39 percent, approximately the same (34 percent) as the signal

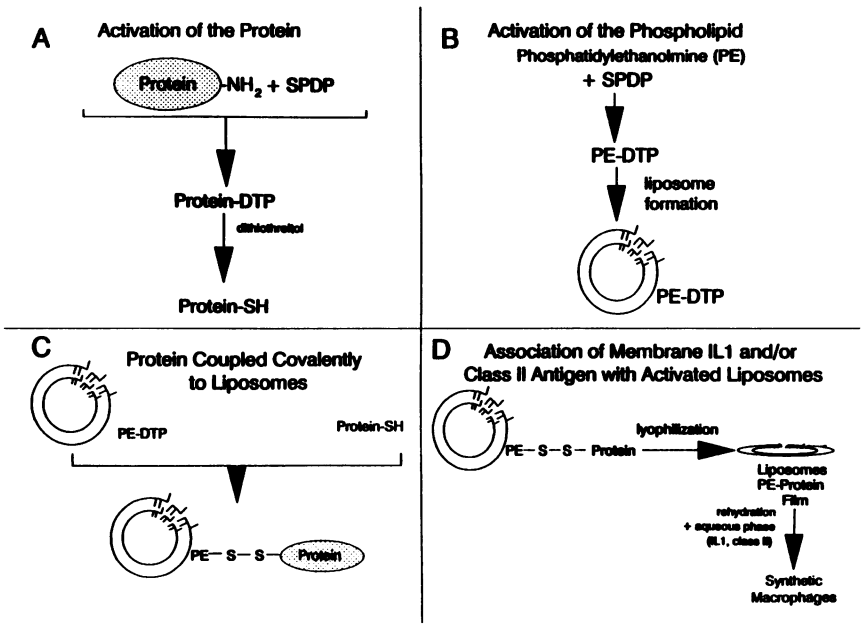

FIG. 1. Schematic representation of the preparation of synthetic macrophages. **A.** Modification of the amino groups of protein antigens to produce free thiol groups. **B.** Preparation of liposomes with modified PE containing a reactive DTP group. **C.** Covalent coupling of the thiol-bearing liposome. **D.** Lyophilization of the protein-coupled liposomes and rehydration with an aqueous phase containing the hydrophobic membrane IL-1 and/or class II antigens to yield synthetic macrophages.

in the absence of conalbumin antigen and IL-1. The combination of I-a^b and membrane IL-1 gave a signal equivalent to the combination of conalbumin/I-a^b/membrane IL-1, 53 percent versus 56 percent, respectively. This result can be explained by the fact that the D10 T-helper clone may be activated by I-a^b alloantigen [15]. When I-a^d was used as the MHC class II antigen to construct the synthetic APC, essentially no proliferation was observed.

D10 Cell Proliferation Kinetics Using Native Conalbumin

We next investigated the influence of membrane IL-1 on the magnitude of the D10 response and on how early the D10 proliferation plateau was reached. For that purpose, synthetic APCs bearing I-a^k/conalbumin/membrane IL-1 were compared with synthetic APCs bearing I-a^k/conalbumin for their ability to stimulate the D10 cells (Fig. 2). When the D10 cells were stimulated by synthetic APC bearing Ia^k/conalbumin/membrane IL-1, the magnitude of the T-cell clone proliferation was higher (120,000 cpm) and the plateau of this response was reached sooner (50 to 55 hours) than when the D10 cells were stimulated by Ia^k/conalbumin macrophages. In the absence of membrane IL-1, the magnitude of the D10 cell response was around 77,000 cpm, and the plateau was reached at 72 hours.

Immune Spleen Cell Proliferation Requirements Using Native Conalbumin

C_3H mice were immunized three times *in vivo* by conalbumin-liposomes, and the immune spleen cells were harvested, separated from splenic macrophages by LeuOMe

TABLE 1
Proliferation of the D10 T-Cell Clone in Response to Conalbumin Antigen

Liposomes Containing[a]	Class II[b] Types	D10 Proliferation[c] cpm	%
Class II + IL-1[d] + Conalbumin[e]	Ia^k	125,000[f]	100
	Ia^b	70,000	56
	Ia^d	2,500	2
Class II + Conalbumin[e]	Ia^k	77,500	62
	Ia^b	48,700	39
	Ia^d	1,250	1
Class II + IL-1[e]	Ia^k	6,250	5
	Ia^b	66,200	53
	Ia^d	1,500	1
Class II	Ia^k	2,000	1
	Ia^b	42,500	34
	Ia^d	1,250	1

[a]Phosphatidylcholine (DPPC: 13.9 μmol), cholesterol (5.6 μmol), and DPPE-DTP (0.7 μmol) were used to prepare the liposomes at a concentration of 500 nmol of lipid/ml of culture medium (50 nmol/well).
[b]The class II MHC molecules were purified as described from a whole cell lysate by lentil lectin chromatography followed by chromatography on respective antibody-sepharose 4B. 18 μg of Ia/μmol of lipid was used.
[c]D10 G4.1 IL-2-dependent murine T-cell clone can proliferate in response to conalbumin presented in the context of syngeneic I-A^k, but also to I-A^b alloantigen and to IL-1 (membrane and soluble forms) when co-cultured with concanavalin A.
[d]Membrane IL-1 was extracted as described in Materials and Methods. Membrane IL-1 was equivalent to 100 total units/μmol of lipid representing the activity of 45 units of IL-1 b (Genzyme)/1,000 μl of assay medium. Membrane IL-1 liposomes did not induce D10 proliferation (2,800 cpm).
[e]Conalbumin (250 μg/ml) was used as antigen, and approximately 40 percent of the starting material was covalently bound to the liposomes; 35.8 μg of conalbumin/μmol of lipid was used. Conalbumin-liposomes were not able to induce D10 proliferation (3,000 cpm).
[f]All values shown were found to have $p < 0.005$ by the student's t test.

treatment plus adherence, and tested for their ability to proliferate in the presence of synthetic APC (Table 2). When synthetic APCs were prepared with Ia^k conalbumin/membrane IL-1, optimal spleen cell proliferation occurred (100 percent = 20,000 cpm). Without membrane IL-1, only 60 percent of the previous signal was observed, and none when liposomes bore only Ia^k (5 percent). Synthetic APC built with both

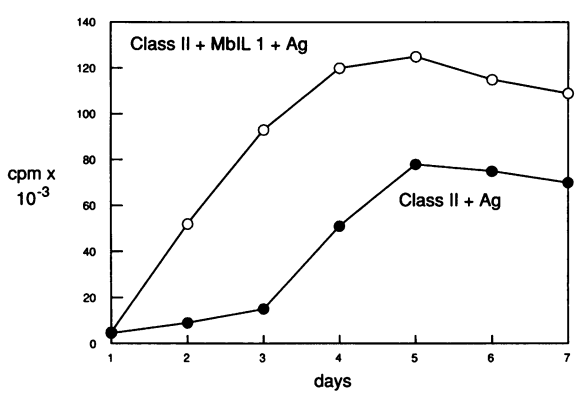

FIG. 2. T-helper cell clone proliferation induced by synthetic macrophages. The D10 IL-2-dependent, antigen-specific murine T-cell line could be activated in culture by encountering antigen (conalbumin) and syngeneic Ia molecules on the surface of APC (C_3H macrophages). Antigen presentation was assayed by measuring D10 proliferation (2×10^4 cells/well) in response to synthetic macrophages bearing either Ia^k/conalbumin/membrane IL-1 (○) or Ia^k/conalbumin (●). Tritiated thymidine incorporation was used to determine D10 proliferation.

TABLE 2
Proliferation of Spleen Cells Sensitized with Conalbumin Antigen

Liposomes Containing[a]	Class II[b] Types	Spleen Cell Proliferation[c]			
		Without Treatment		After Treatment[d]	
		cpm	%	cpm	%
Class II + IL-1[e] + Ag[f]	Ia^k	38,000[g]	100	20,000	100
	I-a^b	15,600	41	3,000	15
	I-a^d	14,800	39	3,400	17
Class II + Ag	I-a^k	32,700	86	12,000	60
	I-a^b	9,500	25	800	4
	I-a^d	8,700	23	1,000	5
Class II + IL-1	I-a^k	9,100	24	5,600	28
	I-a^b	3,800	10	3,000	15
	I-a^d	3,800	10	3,500	17
Class II	I-a^k	1,900	5	1,000	5
	I-a^b	1,500	4	600	3
	I-a^d	1,900	5	600	3

[a]Liposomes were at a concentration of 500 nmol of lipid/ml of culture medium.
[b]Class II molecules were purified as described by lentil lectin chromatography followed by an anti-class II molecule antibody sepharose 4B. 18 μg of Ia/μmol of lipid was used.
[c]C_3H mice were immunized three times *in vivo* with conalbumin antigen presented on the liposome surface.
[d]Spleen macrophages were removed using adherence and LeuOMe (5 mM) treatments.
[e]Membrane IL-1 activity was 100 units/μmol of lipid as measured by the D10 assay. Membrane IL-1 liposomes did not induce spleen macrophage-depleted cell proliferation (950 cpm).
[f]Conalbumin was used as antigen to induce spleen cell proliferation. 35.8 μg of conalbumin/μmol of lipid was used. Conalbumin-liposomes did not induce macrophage-depleted spleen cell proliferation (800 cpm).
[g]All values shown were found to have $p < 0.005$ by the student's t test.

membrane IL-1 and conalbumin antigen with either Ia^b or Ia^d produced a very weak proliferation, 15 percent for Ia^b and 17 percent for Ia^d. This effect was probably due solely to membrane IL-1, since synthetic APC presenting both MHC class II antigen (Ia^b or Ia^d) and membrane IL-1 elicited the same spleen cell proliferation signal, and MHC class II antigen (Ia^b or Ia^d) synthetic APC did not induce a spleen cell response (Table 2).

Other experiments (Table 2) were performed in the presence of splenic macrophages, since the immune spleen cells were not subjected to a lysomotropic agent and adherence. Every time the conalbumin antigen was presented on the liposomes, the proliferation signal was higher in the presence of splenic macrophages than without splenic accessory cells (38,000 cpm when splenic macrophages were not removed versus 20,000 cpm when macrophages were removed). In all other cases, no obvious difference was observed in the degree of proliferation. Most likely, splenic macrophages phagocytose the liposomes bearing the different molecules, among them the conalbumin antigen. The splenic macrophages then process the conalbumin antigen and present it to the splenic T helper cells.

Immune Spleen Cell Proliferation Kinetics Using Native Conalbumin

Spleen cell proliferation in response to synthetic APC bearing Ia^k/conalbumin with or without membrane IL-1 was measured for kinetics and magnitude (Fig. 3). Immune

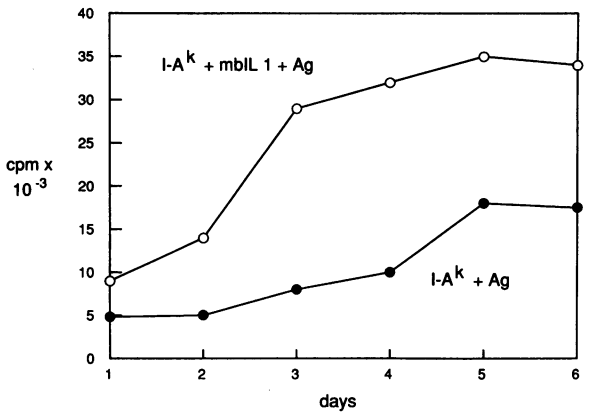

FIG. 3. Kinetics of the spleen cell proliferation induced by synthetic macrophages. C_3H mice were immunized three times *in vivo* with conalbumin liposomes. The spleens were harvested on the fourth week after the third immunization and dissociated in HBSS. The spleen cells were further washed and 2×10^5 cells in RPMI 1640 medium were distributed in a 96-well plate. To determine the minimal concentration of proteins required to induce cell proliferation, serial dilutions of synthetic macrophages were used in triplicate. Spleen cell proliferation induced by lipsomes bearing Ia^k, native conalbumin, and membrane IL-1 (O) is compared with spleen cell proliferation induced by liposomes bearing Ia^k and native conalbumin (●). Concentration of Ia^k was 18 μg/μmol of lipid, the concentration of native conalbumin was 35.8 μg/μmol of lipid, and membrane IL-1 was 10 units/μmol of lipid.

spleen cell proliferation in the presence of membrane IL-1 on the synthetic APC was higher (30,000 cpm with membrane IL-1 versus 16,000 cpm without) and the plateau was more rapidly reached, in three days with membrane IL-1 versus five days without.

DISCUSSION

Our understanding of the function of APC in T-cell activation is steadily expanding. It appears that the first function of APC is the processing and subsequent presentation of antigens in a form appropriate for recognition by T cells [17–19]. It is clear that Ia antigens expressed on the surface of Ia-positive APC provide one signal to T cells necessary for them to recognize either syngeneic or allogeneic Ia alone or Ia in association with conventional non-MHC antigens [20–22]. In addition, it appears that a second signal for T-cell activation is provided by APC through mediators such as IL-1 [23]. To determine the minimal and optimal requirements of T-cell activation, we made synthetic APC by constructing liposomes carrying one or more of a native foreign protein antigen, membrane IL-1, and Ia molecules. We could thus test whether these different artificial macrophages could activate antigen-specific class II restricted T cells in the absence of APC.

In these studies, we used as proliferative cells the D10 G4.1 antigen-specific murine T-cell line or immune spleen cells from mice sensitized three times with conalbumin. D10 is a helper T-cell clone activated by the antigen conalbumin and syngeneic Ia

molecules on surface APC. Alternatively, D10 cells may be activated by APC with a different histocompatibility antigen, C57 BL/6 (H-2^b) spleen macrophages.

When synthetic APCs were constructed using liposomes bearing native conalbumin, membrane IL-1, and Ia^k as class II MHC molecules, a maximum proliferative response was obtained for both the D10 cells (Table 1) and the immune spleen cells (Table 2). When the liposomes bore only native conalbumin and Ia^k, the proliferation intensity was around 40 percent less for the D10 cells (Table 1) and spleen cells (Table 2). In both cases, a greater and more rapid cell response was obtained with synthetic APC bearing native conalbumin/membrane IL-1/Ia^k than with liposomes bearing native conalbumin/Ia^k (Figs. 2 and 3).

When Ia^d was used as class II MHC molecules, no proliferation was detected, even when Ia^d was associated with membrane IL-1 and native conalbumin (Tables 1 and 2). In contrast, Ia^b elicited D10 proliferation (around 35 to 40 percent), even when it was the only molecule at the liposome surface (Table 1). It did not, however, affect spleen cell proliferation, even when it was presented along with membrane IL-1 and conalbumin (Table 2). These Ia^b results are not surprising, since the T-cell clone can proliferate in response to Ia^b alloantigen [15]; however, D10 proliferation when membrane IL-1 was present (53–56 percent), was greater than when it was not (34–39 percent), (Table 1).

From the data of Tables 1 and 2, we can conclude that the recognition of foreign antigen together with class II MHC molecules seems to be the only signal required for the activation of antigen-primed regulatory T cells. Furthermore, "processing" of antigen by APC is not essential for its recognition by T cells. In agreement with Walden et al. [8], the fact that intact protein molecules, when coupled to lipid, can activate T cells indicates that extensive processing is not required to render some proteins immunogenic for T cells. Thus, although antigen degradation certainly takes place in macrophages and also in other cell types [24,25], the belief that regulatory T cells are capable of recognizing *only* processed antigen [26,27] is not universally valid. Clearly, though, the existence of T-cell clones with a preference for partially digested antigens has been demonstrated. [28,29]

When synthetic APC included only Ia^k and native conalbumin, the proliferative response was 40 percent less than that obtained with Ia^k/conalbumin/membrane IL-1 macrophages, showing that membrane IL-1 was complementary but not essential for T-cell activation (Tables 1 and 2). The comparison of these results raises questions about the role of membrane IL-1 in macrophage-T-cell interaction. Kurt-Jones et al. [15] have shown that presentation of foreign protein and alloantigen by fixed macrophages was quantitatively related to the expression of membrane IL-1; i.e., antigen presentation increased with increasing amounts of membrane IL-1. In a different system such as an allogeneic stimulation of cytotoxic T cells using planar membranes, Brian and McConnell [30] demonstrated that H-$2K^k$ reconstituted into vesicles is dependent on added growth factors to stimulate T cells. H-$2K^k$ is recognized by pre-CTL because a significant response occurred only when vesicles contained antigen; however, the requirement for exogenous factors indicates that H-$2K^k$ in planar membranes does not stimulate T cells. By contrast, other studies using H-$2K^k$ reconstituted into vesicles have shown that cytotoxic T-cell allogeneic stimulation was not dependent on added growth factors [31–33]. Krieger et al. [34] demonstrated that the basis for the difference in accessory cell function between resting and activated B cells is not dependent upon co-stimulator production, because the triggering of the

T-cell hybrids used has been shown to be independent of an IL-1 requirement [28,35]. They suggested that the difference in accessory cell functional capacity between resting and activated B cells lies in a difference in their capacity to participate in antigen-independent cellular interactions with T cells, which may be critical for subsequent antigen-specific activation of the T cell. The studies of Lipsky and Rosenthal [36] and Braendstrup et al. [37] have demonstrated that accessory cells and T cells initially interact nonspecifically and that this interaction is subsequently stabilized by antigen-specific interaction. Thus, in the experiments done with native conalbumin, increased concentrations of antigen were required to achieve T-cell activation, because the synthetic APC-T-cell interaction occurs less readily with large proteins.

It appears that the APC-T-cell interaction is a two-step procedure involving recognition, in which membrane IL-1 is not required (even if T-cell stimulation begins), and T-cell activation, in which membrane IL-1, among other factors, is needed. This proposal is in agreement with that by Davis and Lipsky [38], in which T-cell activation requires accessory cells to play at least two distinct roles: the initiation of the response requires a signal conveyed by an intact macrophage, one that cannot be provided by either a macrophage supernatant such as IL-1 [39,40], TNF [41], or PMA [38]. The response is then amplified by additional macrophage or macrophage supernatant factors, in which case PMA can substitute for the macrophage as long as it is supported by a small number of intact accessory cells [38]. More recently, another monocyte/macrophage-derived cytokine, TNF-a, was found to enhance T4 cell proliferation induced by antigen-bearing paraformaldehyde-fixed monocytes [41]. The authors concluded that TNF-a facilitated mature T-cell proliferation and therefore was similar in its action to IL-1 [41].

In Table 2, we have shown that another application of the synthetic macrophage is the presentation of antigen to splenic macrophages, thus acting like an artificial immunogen. The liposomes bearing membrane IL-1, Ia^k, and native conalbumin were probably phagocytosed by the accessory cells, where the antigen was processed and presented to the immune T cells. This tool can be used to increase our understanding of the role of the supermolecular organization of antigen in the expression of its immunogenicity and could help to elucidate how the antigen microenvironment affects the immune recognition process. In addition, it may provide valuable information for designing and preparing appropriate vehicles for synthetic vaccines.

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