Induction of T Cell Anergy by Liposomes with Incorporated Major Histocompatibility Complex (MHC) II/Peptide Complexes

Annemiek J. M. L. van Rensen,[1,2] Leonie S. Taams,[2] Mayken C. Grosfeld-Stulemeyer,[2] Willem van Eden,[2] Daan J. A. Crommelin,[1] and Marca H. M. Wauben[2,3]

Received January 4, 2000; accepted February 29, 2000

Purpose. The aim of this study was to use small unilamellar liposomes with incorporated MHC II/peptide complexes as a carrier system for multivalent antigen presentation to CD4 + T cells.

Methods. Purified peptide pre-loaded MHC II molecules were incorporated into small unilamellar liposomes and tested for their ability to activate A2b T cells. The outcome of T cell activation by such liposomes in the absence of accessory cells was tested via flow cytometry and a T cell anergy assay.

Results. Provided the presence of external co-stimulation, MHC II/peptide liposomes were able to induce proliferation of the A2b T cell clone. More importantly incubation of these T cells with MHC II/peptide liposomes in the absence of co-stimulation did not induce proliferation, however, a MHC/peptide ligand-density dependent down-regulation of the TCR was observed. Interestingly, when T cells after incubation with the MHC II/peptide liposomes were restimulated with their specific antigen in the presence of professional APC, these cells were anergic.

Conclusions. We propose MHC II/peptide liposomes as a novel means to induce T cell anergy. The possibility to prepare 'tailor-made' liposomal formulations may provide liposomes with an important advantage for applications in immunotherapy.

KEY WORDS: liposomes; antigen-presentation; T lymphocytes; anergy; MHC.

INTRODUCTION

Interaction of major histocompatibility complex (MHC) II/peptide complexes with the T cell receptor (TCR) on the cell surface of CD4+ T cells is the key event in T cell activation (1–3). However, activation of CD4+ T cells via MHC-peptide-TCR interaction in the absence of proper co-stimulatory signals can induce a state of T cell anergy (i.e., antigen-specific T cell unresponsiveness) (reviewed in (4)). In this context, the use of 'nonprofessional' or modified antigen-presenting cells (APC) was reported (5,6), as well as the use of immobilized (7) or soluble (8,9) MHC molecules. However, in some cases, apoptosis was induced following the latter protocol (10). The *in vivo* induction of T cell anergy or apoptosis by alternative means of antigen-presentation was also demonstrated (11,12). Although in general it is assumed that immunoregulation via the induction of T cell anergy is based on functional elimination, we and others have shown that anergic T cells can play an active role by suppressing other T cells (5,13). As such the induction of T cell anergy opens novel possibilities for immunotherapy of T cell-mediated (autoimmune) diseases.

For T cell activation the formation of a high-density contact area between T cell and APC, and the necessity of multiple interactions appear to be important (3,14). Also, in the most recent *in vitro* studies employing oligomeric MHC/peptide complexes for passive capturing of specific T cells (15), induction of T cell proliferation (16), or induction of T cell anergy (9), the importance of multivalency is increasingly recognized. In order to present these complexes in a multivalent manner, we proposed a liposomal formulation as a carrier system for MHC II/peptide complexes. Membrane proteins like MHC molecules, containing a hydrophobic transmembrane part can be incorporated into the phospholipid bilayer of liposomes (17). By including multiple protein molecules in the bilayer of fluid state vesicles allowing lateral movement within this bilayer, optimal positioning to make TCR-MHC contact (3) is possible. As each vesicle contains a multitude of peptide loaded MHC-molecules a number of interactions may be simultaneously established, resulting in an (artificial) antigen-presenting vesicle with cell-like surface characteristics. Previously, MHC II-containing liposomes have been employed to study basic aspects of antigen-presentation (7,18). Recently, we have shown that after complying with essential factors like optimal peptide loading of the MHC molecules, functionally active MHC II/peptide liposomes can be prepared (19).

In the present report, we show that well-characterized MHC II/peptide liposomes not only induce antigen-specific IL-2 production of a costimulation-independent T cell hybridoma, but also activate the corresponding costimulation-dependent T cell clone. As MHC II/peptide liposomes are devoid of costimulatory molecules, we were particularly interested in their capacity to induce T cell anergy. In addition, experiments were performed in which external costimulation was provided deliberately in order to be able to fully characterize the T cell activating potential of the MHC II/peptide liposomes.

MATERIALS AND METHODS

Purification of MHC II Molecules

Lewis rat MHC II molecules, RT1.B^L, and RT1.D^L, were purified from the Z1a T cell clone through affinity chromatography using the OX6 and OX17 monoclonal antibody (mAb) respectively (20). Affinity-purified MHC molecules were solubilized in 1% n-β-octyl glucopyranoside (OG) (Sigma).

[1] Department of Pharmaceutics, Faculty of Pharmacy, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands.

[2] Institute of Infectious Diseases and Immunology, Department of Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

[3] To whom correspondence should be addressed at Institute of Infectious Diseases and Immunology, Department of Immunology, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.165, 3508 TD Utrecht, the Netherlands. (e-mail address: m.wauben@vet.uu.nl)

ABBREVIATIONS: APC, antigen-presenting cell; BN, Brown Norway; FITC, fluorescence isothiocyanate; HSP65, 65 kD mycobacterial heat-shock protein; IL-2, interleukin 2; IMDM, iscove's modified Dulbecco's medium; MHC, major histocompatibility complex; MAb, monoclonal antibody; OG, n-ß-octylglucopyranoside; PBS, phosphate-buffered saline; PL, phospholipid; TCR, T cell receptor.

0724-8741/00/0600-0720$18.00/0

Peptides

The RT1.B^L binding peptide 176-190 from mycobacterial 65 kD heat shock protein (HSP65 176–190; EESNTFGLQLELTEG) was synthesized by solid phase Fmoc chemistry (21), purified (>90%) by reversed-phase HPLC, and checked via fast atom bombardment mass spectrometry.

Peptide Loading and Liposome Preparation

Prior to liposome preparation, affinity-purified RT1.B^L or RT1.D^L molecules were incubated with a 100-fold molar excess of HSP65 176–190 for 3 days at pH 5 and 37°C. These conditions were optimal for loading of RT1.B^L (19). Peptide HSP65 176–190 does not bind to RT1.D^L, which is used as control MHC II (data not shown). Liposomes were prepared from egg-yolk phosphatidylcholine (Lipoid), egg-yolk phosphatidylglycerol (Lipoid), and cholesterol (Sigma) (10:1:4, molar ratio) through the method of detergent removal (19). Briefly, appropriate amounts of phospholipids (PL), cholesterol, and fluorescence-labeled PL (Texas Red phosphatidylethanolamine (Molecular Probes) final concentration of 0.5 mole% of total PL) were dissolved in ethanol (absolute) and mixed. A lipid film was obtained through rotary evaporation under reduced pressure at 40–50°C, followed by a N_2 flush. The lipid film was dispersed in PBS (pH 7), containing 1% OG and peptide-preloaded MHC II molecules (initial MHC to PL molar ratio ranging from 1/1200 to 1/4000). Liposomes were formed via controlled dilution with detergent-free PBS, followed by two subsequent ultracentrifugation steps (230,000xg, 90 min., 4°C). Final liposome pellets were redispersed in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) at a phospholipid concentration of 8–16 mM (residual OG: < 0.01% as determined by gas chromatography (19)). A control liposome formulation was included to check the effect of potential trace amounts of free peptide in the assays. For this purpose, the lipid film was dispersed in a peptide/OG solution without MHC II, or in the presence of the control MHC II, RT1.D^L, and liposomes were prepared as described previously.

Characterization of MHC II/Peptide Liposomes

Mean particle sizes of the liposomes were determined through dynamic light scattering with a Malvern 4700 system, using a 25 mW Ne-He laser and the automeasure version 3.2 software (Malvern Ltd.). For refractive index and viscosity, the values for pure water were used. The particle size distribution was reflected in the polydispersity index, ranging from 0.0 for monodisperse to 1.0 for polydisperse dispersions. MHC II/peptide liposomes were typically 0.10–0.12 μm in diameter with narrow particle size distributions (polydispersity index: 0.1–0.2). The relative MHC II incorporation efficiency was checked through flow cytometric analysis of the Texas Red positive liposomes using a FACSCAN (Becton Dickinson) (19). Due to the low sensitivity of generally used protein assays, absolute MHC II incorporation efficiencies were only determined for the highest ligand density liposome formulation (initial MHC:PL = 1/1200). For this, the spot-protein assay of Minamide and Bamberg was employed (22), as described before (19). The final MHC:PL ratio for this formulation was at least 1/1700 (M:M) (MHC II incorporation efficiency: 70% (19)). Assuming unilamellarity, this corresponded to 200–300 MHC II molecules per liposome (data not shown).

In Vitro Proliferation of the A2b T Cell Clone

The A2b T cell clone was generated and maintained as described before (23). Proliferation of the A2b T cell clone was measured in a lymphocyte stimulation assay in flat-bottomed microtiter plates (Costar) in triplicate cultures. Each well contained 2×10^4 T cells, 1×10^6 irradiated (3000 rads) thymocytes, and a dose range of liposomes or soluble specific peptide in IMDM supplemented with 2 mM L-glutamine, 50 μM β-mercaptoethanol, 2% heat inactivated rat serum, and antibiotics. Both MHC matched (Lewis) and MHC non-matched (Brown-Norway (BN) thymocytes were used as accessory cells to provide external costimulation. Following 72 hours of culturing, cells were pulsed with [^{3}H]-thymidine (0.4 μCi/well; specific activity 1 Ci/mmol, Amersham) for 16–18 hours. Cells were harvested on fibreglass filters and [^{3}H]-thymidine incorporation was measured using a scintillation counter (Wallac Oy).

Flow Cytometric Analysis of Cell Surface Markers

For the flow cytometric analysis of TCR expression, A2b T cells were washed and incubated in 4% rat serum for at least 30 minutes. Subsequently, cells were stained for 30 minutes on ice with mAb R73 (anti-αβTCR rat, mouse IgG1) or the relevant isotype control (UD15, anti-chloramphenicol, mouse IgG1), followed by incubation with goat anti-mouse Ig-FITC (Becton-Dickinson). Cells were analyzed using a FACSCAN apparatus (Becton Dickinson). Cells were checked for apoptosis through staining with FITC-conjugated Annexin V, according to the manufacturer's instructions (Apoptest-FITC kit, Nexins Research B.V.). A positive control for the induction of apoptosis, as suggested by the manufacturer (*i.e.* incubation of the cells in 3% formaldehyde/PBS), was included.

The clustering of MHC II/peptide liposomes with the A2b T cells was monitored via flow cytometric analysis of T cell-associated Texas Red fluorescence.

A2b T Cell Anergy Assay

T cell anergy induction of the A2b T cell clone was assayed as described previously (5), with minor modifications. A2b T cells (3×10^6/ml) were incubated with an optimal concentration of MHC II/peptide liposomes (0.8 mM final PL concentration; MHC:PL ratio ranging from 1:1200 to 1:4000 (M:M)) for 4 hours at 37°C and 5% CO_2 in supplemented IMDM and 2% heat-inactivated rat serum. Viable cells were collected via Ficoll-Isopaque gradient centrifugation, rested for 3 days to allow re-expression of the TCR, again collected via Ficoll-Isopaque gradient centrifugation, and then tested for proliferative responses. During the lymphocyte stimulation assay, 24-hour cell culture supernatants were collected and tested for IL-2 activity in a CTLL16 assay (19). A2b responsiveness to IL-2 was tested in the lymphocyte stimulation assay by adding 10 U/ml recombinant human IL-2 (Pharmingen).

RESULTS

MHC II/Peptide Liposomes Induce Proliferation of the A2b T Cell Clone in the Presence of Co-Stimulation

Previously, we showed that (RT1.B^L/HSP65 176-190)-liposomes can serve as antigen-presenting vesicles by specifically inducing a dose dependent IL-2 production of the A2b

hybridoma, which was as efficient as normal peptide/APC stimulation (19).To study this, affinity-purified peptide-preloaded MHC II molecules were reconstituted into liposomes at various initial MHC:phospholipid (PL) ratios, and the effects were tested in the A2b T cell hybridoma assay. To extend the findings obtained with the hybridoma to a less artificial T cell system, the (RT1.B^L/HSP65 176-190)-liposomes were tested for their T cell activating capabilities using the A2b T cell clone. In contrast to the A2b T cell hybridoma, this clone is dependent on co-stimulation for optimal activation. Consequently, (RT1.B^L/HSP65 176-190)-liposomes alone were not able to induce proliferation in a standard lymphocyte stimulation test (data not shown). In order to use T cell proliferation as a readout system for A2b T cell clone activation in response to the (RT1.B^L/HSP65 176-190)-liposomes, it was therefore necessary to provide external (trans-) co-stimulation during the cell culture assay (24). For this purpose, MHC matched or non-matched thymocytes were added as accessory cells. Figure 1A shows the peptide dose-response curves of the A2b T cell clone in the presence of APC. When Lewis rat derived thymocytes (MHC matched) were added, a normal dose-response curve was obtained, whereas, in the presence of Brown Norway (BN) rat derived thymocytes (MHC non-matched) only background proliferation was observed. This background proliferation was most likely the result of T cell-T cell presentation of peptide by the MHC II-positive A2b T cell clone (25). In the presence of external (trans-) co-stimulation, (RT1.B^L/HSP65 176-190)-liposomes were capable of triggering proliferative responses of the A2b T cell clone. Figure 1B demonstrates that A2b T cells proliferate in response to the (RT1.B^L/HSP65 176-190)-liposomes in the presence of the MHC matched (Lewis) accessory cells. Furthermore, although to a lesser extent, proliferation was also observed in the presence of the non-matched (BN) accessory cells. Since the BN rat derived cells are not capable of presenting the HSP65 176-190 peptide to the A2b T cell (Fig. 1A), these cells only contributed in providing co-stimulation, leaving the antigen-presentation exclusively to the MHC II/peptide liposomes. Control liposome formulations made in the presence of HSP65 176-190/OG solution without MHC II, did not induce A2b T cell proliferation (Fig. 1B). To compare Figs. 1A and 1B we estimated the amount of HSP65 176-190 present during the assay. Assuming 100% MHC II incorporation efficiency and 100% peptide loading of MHC II, at maximum 0.37 μg/ml HSP65 176-190 peptide was present at the highest liposome concentration tested (Fig. 1B). However, the peptide concentration was probably lower as the absolute MHC incorporation efficiency was approximately 70% (See Materials & Methods). Moreover, although we used the optimal conditions for peptide-MHC loading, the maximum achievable percentage of peptide loaded MHC II molecules most likely varies between 20–60% (19,26). Taking into account 70% MHC II incorporation efficiency and 20–60% peptide loading of MHC II, the HSP65 176-190 concentration at the highest liposome concentration varies between 0.05–0.15 μg/ml.

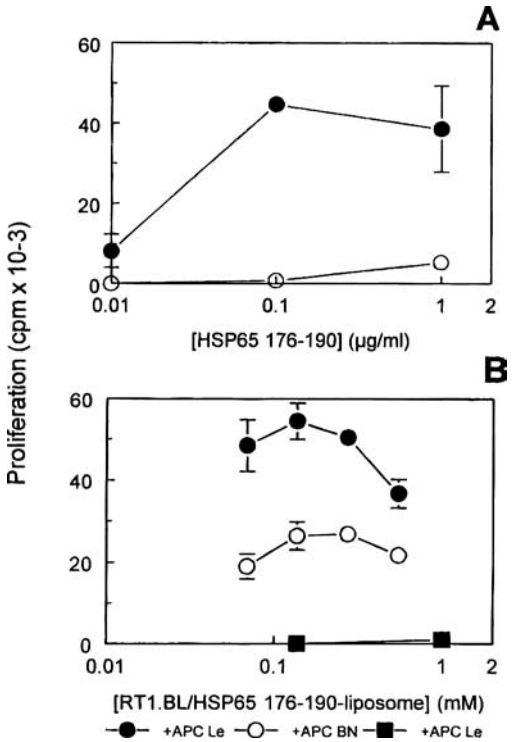

Fig. 1. Proliferation of the A2b T cell clone in response to A: soluble peptide, in the presence of irradiated thymocytes as APC; or B: (RT1.B^L/HSP65 176-190)-liposomes (initial molar ratio PL:MHC = 1:4000, circular symbols), or control liposomes (HSP65 176-190 without MHC II, square symbols) in the presence of Lewis rat-derived thymocytes (MHC-matched) or Brown-Norway rat-derived thymocytes (MHC non-matched). On the X-axis of 1A, the peptide concentration is indicated in μg/ml, while in 1B the phospholipid concentration is indicated in mM. Proliferative responses are expressed in cpm as means of triplicate cultures ± SD. The background proliferation of clone A2b in medium in the absence or presence of thymocytes was < 1000 cpm. The results are a representative of at least 3 independent experiments.

Incubation of the A2b T Cell Clone with MHC II/Peptide Liposomes in the Absence of Co-Stimulation Induces TCR Downregulation

To study the effect of (RT1.B^L/HSP65 176-190)-liposomes on the TCR expression, liposomes were prepared with different MHC II densities on their surface (MHC:PL ranging from 1/1200–1/4000). A2b T cells were incubated with these liposomes ([PL] = 0.8 mM) for 4 hours at 37°C and 5% CO_2. Following incubation, A2b cells were washed and analyzed via flow cytometry. After 4 hours of incubation, the clustering of Texas Red-labeled (RT1.B^L/HSP65 176-190)-liposomes with the A2b T cells was clearly visible by the T cell-associated red fluorescence, as compared to the (background) fluorescence after incubation with the control liposome formulation (Texas Red-labeled liposomes made in the presence of HSP65 176-190/OG without MHC II) (Fig. 2). The degree of liposome-T cell clustering furthermore strongly depended on the MHC II density on the liposomes. Interestingly, already at this early stage, the expression of the TCR on the A2b T cells was downregulated (Fig. 3A). No cell-associated Texas Red could be detected after incubation with the anti-TCR mAb (R73), ensuring that (possibly residual) liposomes did not hinder proper analysis of TCR downregulation. Also, TCR downregulation was ligand-density dependent and most obvious after incubation with liposomes with the highest MHC II/peptide density. TCR downregulation was transient and the expression of TCR had returned to control levels after 3 days recovery (Fig. 3B). Control liposomes

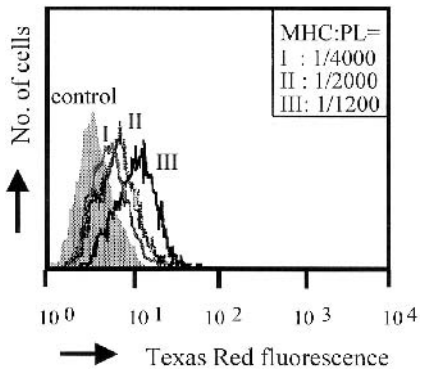

Fig. 2. Density-dependent clustering of (RT1.B^L/HSP65 176-190)-liposomes with A2b T cells. A2b T cells were incubated with the different liposome formulations for 4 hours at 37°C, washed and analyzed via flow cytometry for cell-associated Texas Red fluorescence.

containing no MHC II did not induce TCR downregulation (Fig. 3A, 3B).

Activation of the A2b T Cell Clone by MHC II/Peptide Liposomes Does Not Lead to Apoptosis but Induces a State of T Cell Anergy

Next, we analyzed whether incubation of the A2b T cell clone with the MHC II/peptide liposomes induced apoptosis. However, by using the standard Annexin V apoptosis assay (27), we did not find any indication for apoptosis induction (data not shown). Subsequently, we studied the effect of MHC II/peptide liposomes incubation on antigen-responsiveness of the A2b cells. Following the pre-incubation period, T cells were recovered for 3 days to allow re-expression of the TCR (Fig. 3B), and subsequently rechallenged in a standard T cell proliferation assay in the presence of professional APC.

Interestingly, when A2b T cells after pre-incubation with the liposomes were rechallenged these cells were anergic, i.e. they had lost the capacity to proliferate in response to their specific antigen (Fig. 4A). Moreover, no IL-2 was detected in the 24 hour-supernatants from cells that were preincubated with the MHC II/peptide liposome formulations and restimulated with the specific peptide in the presence of APC (Fig. 4B). The typical anergic phenotype could furthermore be deduced from the increased responsiveness to IL-2 of anergic cells as compared to the control cells (Fig. 4A). These anergic T cells had a normal expression of the TCR at the time of antigenic restimulation which is represented in Fig. 3B, and these cells were still capable of responding to a mitogenic stimulus, as indicated in the legend of Fig. 4. Although the MHC II/peptide density on the liposomes influenced the amount of cell-associated liposomes as analyzed through Texas Red fluorescence (Fig. 2) and the degree of TCR downregulation (Fig. 3A), complete T cell unresponsiveness was induced by all 3 formulations.

To exclude an effect of any non-MHC liposome-associated peptide on the induction of T cell anergy, control liposomes were prepared with the control MHC II (RT1.D^L) in the presence of HSP65 176-190/OG and compared to the (RT1.B^L/HSP65 176-190)-liposomes. Since the HSP65 176-190 peptide does not bind to RT1.D^L, only non-specifically associated peptide will be present in this formulation. As can be seen in Fig. 4C, the (RT1.B^L/HSP65 176-190)-liposomes again induced a marked reduction in antigen-specific T cell responsiveness (>60%), while the control (RT1.D^L /HSP65 176-190)-liposome preparation had no effect on T cell responsiveness. Furthermore, while the induction of IL-2 hyperresponsiveness was evident for the relevant formulation, it was absent in case of the RT1.D^L liposomes.

DISCUSSION

We had reported that well-characterized MHC II/peptide liposome formulations can induce a dose-dependent antigen-specific IL-2 production of a costimulation-independent T cell

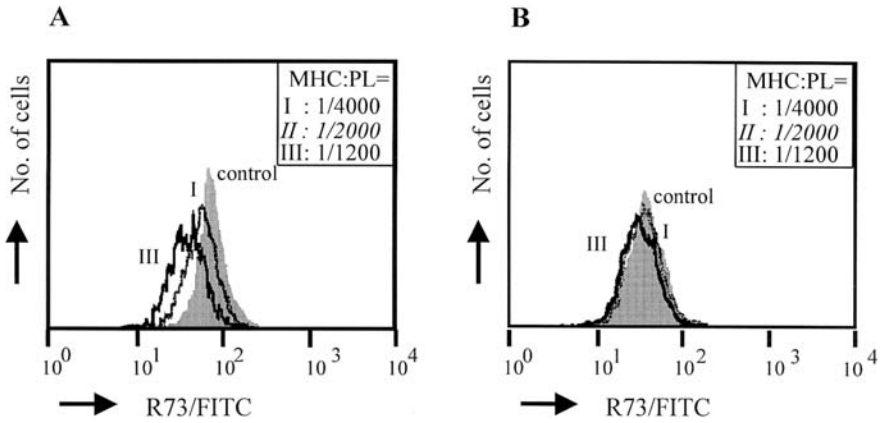

Fig. 3. Flow cytometric analysis of TCR expression of A2b T cells, A: directly after 4 hours incubation with control (HSP65 176-190 without MHC) or (RT1.B^L/HSP65 176-190) liposomes and B: 3 days after the initial incubation period. Before analysis, cells were washed and stained with mouse anti-rat αβ TCR (R73) and FITC-goat anti-mouse Abs. For clarity reasons, the curves of formulation II (intermediate ligand density) were left out. TCR expression after incubation with this formulation was always in between that of conditions I and III. TCR expression of A2b T cells after incubation with control liposomes did not differ from TCR expression of non-stimulated cells.

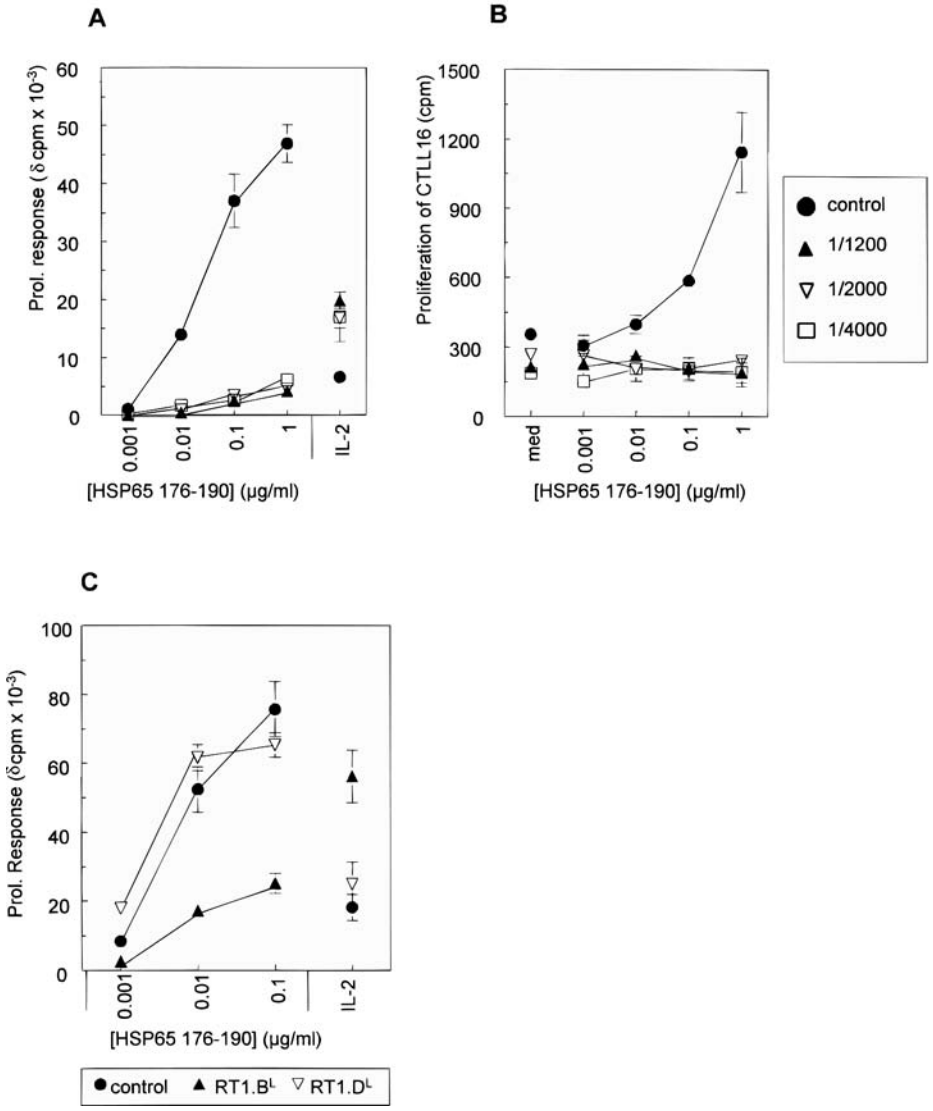

Fig. 4. Induction of T cell anergy in A2b T cells by (RT1.B^L/HSP65 176-190)-liposomes. A2b T cells were incubated for 4 hours with liposomes with different MHC II/peptide complex densities on their surface (MHC:PL ranging from 1:1200–1:4000). Before restimulation, cells were recovered for 3 days. A: Proliferation of the A2b T cells after preincubation with liposomes (1:1200–1:4000) or medium (control) in response to the specific peptide and to IL-2 (10 U/ml) in the presence of irradiated Lewis thymocytes as APC. Proliferation was assayed via [^{3}H]-thymidine incorporation. Responses to Concanavalin A were >45,000 cpm in all conditions. B: IL-2 production of the A2b T cells after preincubation with liposomes or medium, in response to the specific peptide in the presence of APC. IL-2 production was determined via proliferation of the IL-2 dependent CTLL16 cell line (See Materials & Methods). C: No anergy induction in A2b T cells by (RT1.D^L/HSP65 176-190)-liposomes. Proliferation of A2b T cells was assayed after preincubation with (RT1.D^L/HSP65 176-190)-liposomes and compared to pre-incubation with (RT1.B^L/HSP65 176-190)-liposomes (MHC:PL 1:4000) or medium (control). The results were reproduced in 3 independent experiments.

hybridoma (19). In the present paper, the use of MHC II/peptide liposomes as antigen-presenting vesicles was further extended to activation of the corresponding T cell clone. As expected, MHC II/peptide liposomes alone did not induce proliferation of this costimulation-dependent clone. However, proliferation of the T cell clone could be achieved when accessory cells were added to provide (trans-) co-stimulation. In the presence of MHC-matched accessory cells T cell activation by the MHC II/peptide liposomes was restored up to at least a similar proliferation as compared to peptide stimulation in the presence of professional APC. Although we can not fully exclude the possibility of endocytosis of the liposomes by the MHC-matched accessory cells and subsequent antigen-presentation by these cells, the experiment with the MHC non-matched cells demonstrated that such a mechanism does not account for the main route of induction of A2b proliferation. The fact that

proliferation in the presence of the irrelevant accessory cells was lower as compared to the MHC matched accessory cells is most likely due to the suboptimal co-stimulatory capacities of these cells (24).

It is interesting that although no proliferation could be measured when the A2b T cell clone was incubated with MHC II/peptide liposomes in the absence of accessory cells, a ligand-density dependent liposome-T cell clustering was observed via flow cytometric analysis. Moreover, the TCR was downregulated following such an incubation step, being indicative of the fact that MHC II/peptide liposomes could trigger an early event in T cell stimulation. TCR downregulation has been described as an early event in T cell activation (28), which can be dissociated from the events required for full T cell activation (29).

Others have reported various outcomes of T cell activation obtained via protocols employing soluble or immobilized MHC II/peptide complexes, ranging from T cell unresponsiveness (8,9), to apoptosis (10). We show here that T cell activation by MHC II/peptide liposomes in the absence of co-stimulation had a profound effect on the subsequent antigen-responsiveness of the A2b T cells. After restimulation with professional APC and peptide, complete T cell unresponsiveness was observed in such T cells. This unresponsiveness bore the typical characteristics of T cell anergy as defined previously (4,5).

Although we can not fully exclude the possible presence of trace amounts of non-liposome incorporated MHC II molecules, or aggregates thereof, which may be involved in lymphocyte stimulation or anergy induction, the extremely efficient MHC II incorporation as well as removal of residual detergent, make it highly unlikely that non-liposome incorporated MHC II molecules will be present to a sufficiently high amount to account for these effects. Moreover, it has been described that at least the dimerization of MHC II molecules is needed for T cell activation (9). However, due to the toxicity of the detergent, we were unable to perform the ultimate test to check whether detergent-solubilized MHC II molecules can activate and anergize T cells.

The possibility of inducing antigen-specific T cell anergy by MHC II/peptide liposomes offers interesting opportunities. Compared to the use of soluble monomeric or oligomeric MHC II/peptide complexes, antigen-presentation by MHC II/peptide liposomes occurs in a highly multivalent way and the ligand-density can be controlled. Although we do not proof the necessity of multivalency for anergy induction in the present paper, this may be a very crucial factor, since multivalency at the interaction site of TCR and MHC/peptide complex is considered important for T cell activation (15,16). Furthermore, because of the fluidity of the bilayer, the MHC II/peptide complexes are not fixed in position, which may be important in the formation of an activation patch between T cell and APC (3). Apart from these aspects, liposomes may be interesting for *in vivo* applications because of the possibilities of modifying their pharmacokinetic behaviour to suit needs with respect to tissue distribution or half-life (30). As such, liposomes with incorporated MHC II/peptide complexes can be an attractive means for induction of T cell anergy, and may create novel openings for antigen-specific immunotherapy of T cell mediated diseases.

ACKNOWLEDGMENTS

This work was sponsored by N.V. Organon, Oss, The Netherlands. The research of M. H. M. Wauben has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences. The authors wish to thank Dr. R. van der Zee and A. Noordzij for peptide synthesis, and C. Versluis for FAB-MS peptide analysis (Biomolecular Mass Spectrometry, Bijvoet Center, Utrecht University). Dr. G. Verheijden (N.V. Organon, Oss, the Netherlands) is acknowledged for helpful discussions, and Prof. I. R. Cohen (Weizmann Institute, Rehovot, Israel) for the gift of T cell clone A2b.

REFERENCES

1. J. D. Ashwell and R. H. Schwartz. T-cell recognition of antigen and the la molecule as a ternary complex. *Nature* **320**:176–179 (1986).
2. A. Viola and A. Lanzavecchia. T cell activation determined by T cell receptor number and tunable thresholds. *Science* **273**:104–106 (1996).
3. A. Grakoui, S. K. Bromley, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, and M. L. Dustin. The immunological synapse: A molecular machine controlling T cell activation. *Science* **285**:221–227 (1999).
4. R. H. Schwartz. Models of T cell anergy: is there a common molecular mechanism? *J. Exp. Med.* **184**:1–8 (1996).
5. L. S. Taams, A. J. M. L. van Rensen, M. C. M. Poelen, C. A. C. M. van Els, A. C. Besseling, J. P. A. Wagenaar, W. van Eden, and M. H. M. Wauben. Anergic T cells actively suppress T cell responses via the antigen presenting cell. *Eur. J. Immunol.* **28**:2902–2912 (1998).
6. M. K. Jenkins and R. H. Schwartz. Antigen presentation by chemically modified splenocytes which induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med.* **165**:302–319 (1987).
7. H. Quill and R. H. Schwartz. Stimulation of normal inducer T cell clones with antigen presented by purified la molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. *J. Immunol.* **138**:3704–3712 (1987).
8. M. W. Nicolle, B. Nag, S. D. Sharma, N. Willcox, A. Vincent, D. J. Ferguson, and J. Newsom-Davis. Specific tolerance to an acetylcholine receptor epitope induced in vitro in myasthenia gravis CD4+ lymphocytes by soluble major histocompatibility complex class II-peptide complexes. *J. Clin. Invest.* **93**:1361–1369 (1994).
9. J.-P. Abastado, Y.-C. Lone, A. Casrouge, G. Boulot, and P. Kourilsky. Dimerization of soluble major histocompatibility complex-peptide complexes is sufficient for activation of T cell hybridoma and induction of unresponsiveness. *J. Exp. Med.* **182**:439–447 (1995).
10. B. Nag, T. Kendrick, S. Arimilli, S. C. T. Yu, and S. Sriram. Soluble MHC II-peptide complexes induce antigen-specific apoptosis in T cells. *Cell. Immunol.* **170**:25–33 (1996).
11. N. Zavazava and M. Kronke. Soluble HLA class I molecules induce apoptosis in alloreactive cytotoxic T lymphocytes. *Nature Med.* **2**:1005–1010 (1996).
12. S. Bourdoulous, E. Beraud, C. Le Page, A. Zamora, A. Ferry, D. Bernard, A. D. Strosberg, and P. O. Couraud. Anergy induction in encephalitogenic T cells by brain microvessel endothelial cells is inhibited by interleukin-1. *Eur. J. Immunol.* **25**:1176–1183 (1995).
13. G. Lombardi, S. Sidhu, R. Batchelor, and R. Lechler. Anergic T cells as suppressor cells in vitro. *Science* **264**:1587–1589 (1994).
14. S. Valitutti and A. Lanzavecchia. Serial triggering of TCRs: a basis for the sensitivity and specificity of antigen recognition. *Immunol. Today* **18**:299–304 (1997).
15. F. Crawford, H. Kozono, J. White, P. Marrack, and J. Kappler. Detection of antigen-specific T cells with multivalent soluble class II MHC covalent peptide complexes. *Immunity* **8**:675–682 (1998).
16. J. D. Altman, P. A. H. Moss, P. J. R. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. Phenotypic analysis of antigen-specific T lymphocytes. *Science* **274**:94–96 (1996).
17. A. Chakrabarti, J. Matko, N. A. Rahman, B. G. Barisas, and

M. Edidin. Self-association of class I major histocompatibility complex molecules in liposome and cell surface membranes. *Biochemistry* **31**:7182–7189 (1992).
18. T. H. Watts, A. A. Brian, J. W. Kappler, P. Marrack, and H. M. McConnell. Antigen presentation by supported planar membranes containing affinity-purified I-Ad. *Proc. Natl. Acad. Sci. USA* **81**:7564–7568 (1984).
19. A. J. M. L. van Rensen, M. H. M. Wauben, M. C. Grosfeld-Stulemeyer, W. van Eden, and D. J. A. Crommelin. Liposomes with incorporated MHC class II/peptide complexes as antigen-presenting vesicles for specific T cell activation. *Pharm. Res.* **16**:198–204 (1999).
20. I. Joosten, M. H. Wauben, M. C. Holewijn, K. Reske, L. O. Pedersen, C. F. Roosenboom, E. J. Hensen, W. van Eden, and S. Buus. Direct binding of autoimmune disease related T cell epitopes to purified Lewis rat MHC class II molecules. *Int. Immunol.* **6**:751–759 (1994).
21. J. M. Steward and J. D. Young. *Solid Phase Peptide Synthesis.* Pierce Chemical, Rockford, IL, 1984.
22. L. S. Minamide and J. R. Bamburg. A filter paper dye-binding assay for quantitative determination of protein without interference from reducing agents or detergents. *Anal Biochem* **190**:66–70 (1990).
23. J. Holoshitz, A. Matitiau, and I. R. Cohen. Arthritis induced in rats by cloned T lymphocytes responsive to Mycobacteria but not to collagen type II. *J. Clin. Invest.* **73**:211–215 (1984).
24. L. Ding and E. M. Shevach. Activation of CD4+ T cells by delivery of the B7 costimulatory signal on bystander antigen-presenting cells (trans-costimulation). *Eur. J. Immunol.* **24**:859–866 (1994).
25. C. P. M. Broeren, M. H. M. Wauben, M. A. Lucassen, M. van Meurs, P. J. S. van Kooten, C. J. P. Boog, E. Claassen, and W. van Eden. Activated rat T cells synthesize and express functional major histocompatibility class II antigens. *Immunology* **84**:193–201 (1995).
26. B. Nag, H. Garrett Wada, S. V. Deshpande, D. Passmore, T. Kendrick, S. D. Sharma, B. R. Clark, and H. M. McConnell. Stimulation of T cells by antigenic peptide complexes with isolated chains of major histocompatibility complex class II molecules. *Proc. Natl. Acad. Sci.* **90**:1604–1608 (1993).
27. I. Vermes, C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Meth.* **184**:39–51 (1995).
28. S. Valitutti, S. Muller, M. Cella, E. Padovan, and A. Lanzavecchia. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* **375**:148–151 (1995).
29. M. Salio, S. Valitutti, and A. Lanzavecchia. Agonist-induced T cell receptor down-regulation: molecular requirements and dissociation from T cel activation. *Eur. J. Immunol.* **27**:1769–1773 (1997).
30. G. Storm, and D. J. A. Crommelin. Liposomes: Quo vadis? *PSTT* **1**:19–31 (1998).