FEBS Letters 478 (2000) 178–182 FEBS 23967

Generation of both MHC class I- and class II-restricted antigenic peptides from exogenously added ovalbumin in murine phagosomes

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Received 16 June 2000; accepted 6 July 2000

Edited by Marco Baggiolini

Abstract The phagosome fraction derived from a murine macrophage cell line (J774.1), which had internalized ovalbumin (OVA)-coated latex beads, was isolated. The peptides recovered from the phagosome fraction were separated on reverse phase HPLC and each fraction was analyzed for the content of either major histocompatibility complex (MHC) class I- or class II-restricted OVA-derived peptide. Both peptides were detected in the phagosome fraction after less than 15 min of internalization. It was also indicated that phagosomes degrade OVA protein into both MHC class I- and class II-restricted antigenic peptides by employing the same types of cathepsins. Furthermore, the results suggest that the MHC class I-restricted peptide rapidly exits from the phagosome to the cytosol. These findings illustrate a potential role for phagosomes not only in MHC class II-restricted but also in MHC class I-restricted exogenous antigen presentation pathways. Our results also point to the vital role of phagosomes in non-cytosolic antigen presentation pathway, in which further degradation of antigens by the proteasome is dispensable. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Macrophage; Phagosome; Major histocompatibility complex; Antigen presentation

1. Introduction

In the initial step of immune responses, antigen processing and presentation by antigen presenting cells (APCs) are crucial. In general, this step has been classified into two distinct pathways [1–3]. Exogenous antigens are processed through the major histocompatibility complex (MHC) class II-restricted pathway, in which antigens are internalized by APC and degraded by proteases in their acidic vesicular compartments, such as the endosome and the lysosome. The generated peptides are loaded onto MHC class II molecules under the influence of MHC DM and DO molecules [4], and are then transported to the cell surface for recognition by $CD4^+$ T cells. On the other hand, endogenous antigens are processed through the MHC class I-restricted pathway, in which cytoplasmic proteins are degraded by the proteasome and the resulting peptides which are generated are transported into the endoplasmic reticulum (ER) by a membrane-associated peptide transporter (TAP), and assembled into complexes with nascent MHC class I molecules. The peptide–MHC class I complexes are then transported to the cell surface for recognition by $CD8^+$ T cells. Recent findings, however, have challenged the above strict segregation of MHC class I and class II peptide loading pathways [5,6]. One of the most intriguing exceptions is the fact that exogenous particulate antigens, including microbes, are internalized and processed by phagocytes, and the resulting antigenic peptides which are generated are presented by means of MHC class I molecules to $CD8^+$ T cells [7–11]. Although this novel pathway may play an important role in the immune surveillance of a host, little is known relative to where and how antigenic peptides are generated and encountered MHC class I molecules. The results of early studies suggested that antigens may be transferred from vacuolar compartments (e.g. phagolysosomes) to the cytosol, thereby following the conventional MHC class I pathway which is highly dependent on both the proteasome and TAP [10]. In contrast, other studies have concluded that there exists a non-cytosolic pathway for the presentation of exogenous antigens by MHC class I molecules. For example, the presentation of bacteria-associated or bead-bound ovalbumin (OVA) is not affected by the presence of brefeldin A, an inhibitor of protein exocytosis from the ER [7,9]. In another study, the MHC class I presentation of viral antigen was found to be unaffected by a TAP mutation [12]. In both pathways, internalized exogenous antigens are presumably degraded first in vacuolar compartments, and then loaded onto MHC class I molecules. In the cytosolic pathway, antigenic proteins are not necessarily degraded into small peptides, which are ready to bind to MHC class I molecules in phagosomes, since the proteasome resides in the cytosol for further degradation. However, in the non-cytosolic pathway, it is an advantage for the APC to generate 'ready to bind' peptides in the phagosome, regardless of where they bind to MHC class I molecules.

The present study describes an attempt to determine whether a phagosome is capable of degrading a protein antigen (OVA) into small peptides which are able to bind to either MHC class I or class II molecules without the need for further degradation. Our results show that both types of peptides can be simultaneously generated in the phagosome and the same types of cathepsins are involved in the digestion of both types of peptides. Furthermore, it was found that the generated MHC class I-restricted antigenic peptides rapidly exit the phagosome to the cytosol. The role of phagosomes in the immune surveillance for exogenous pathogens is discussed from the viewpoint of possible applications for the development of vaccines.

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Abbreviations: APC, antigen presenting cell; MHC, major histocompatibility complex; ER, endoplasmic reticulum; TAP, transporter associated with antigen presentation; OVA, ovalbumin

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PII: S0014-5793(00)01849-4

2. Materials and methods

2.1. Mice and cells

Female BALB/c mice and C57BL/10 mice were purchased from Japan SLC Inc. (Shizuoka, Japan). A murine macrophage cell line, J774.1 was maintained in RPMI 1640 containing 10% heat-inactivated fetal calf serum, 1% glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. An $OVA_{323-339}$-specific, I-A^d-restricted T cell clone (DOT.1) was established in our laboratory from a strain of transgenic mice, which express T cell receptors from the OVA-specific DO11.10 T hybridoma [13] and was generously provided by Dr. Shimizu (Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan). An $OVA_{257-264}$ (SIINFEKL)-specific, H-$2K^b$-restricted T cell hybridoma (CD8OVA1.3) [7] was generously provided by Dr. Harding (Case Western Reserve University, Cleveland, OH, USA). An IL-2-dependent T cell clone, CTLL-2, was utilized for determining the amount of IL-2 produced by the above mentioned T cell clone or the T cell hybridoma upon stimulation.

2.2. Reagents

Latex beads (0.8 μm, Sigma, St. Louis, MO, USA) were non-covalently conjugated with OVA by incubation with 3.2 mg/ml of OVA (Sigma, St. Louis, MO, USA) in citrate buffer (pH 4.2) as described by Ramachandra et al. [14]. E-64-d and CA-074, prepared by M. Murata et al., were donated by Dr. Hanada, Taisho Pharmaceutical Co. (Saitama, Japan). Pepstatin A was purchased from Sigma (St. Louis, MO, USA). The gel filtration column was obtained from Pharmacia; the reverse phase high performance liquid chromatography (HPLC) column (TSK-80TM) was from Tosoh (Tokyo, Japan).

2.3. Isolation of phagosome fraction

Phagosomes were isolated by flotation on sucrose gradients as described by Desjardins et al. with minor modifications. [15]. Phagosomes were formed by the internalization of latex beads (10% suspension) diluted 1:200 in culture medium (4 ml/dish) at 37°C for 15 min. The cells were then washed with phosphate-buffered saline (PBS) (3×10 min) at 4°C on a rocker, followed by a chase period (from 15 to 45 min) in medium without beads at 37°C. All pulses and chases were performed in an atmosphere of 5% CO_2. After the internalization of the latex beads, the cells were washed with cold PBS (three times×5 min) and scraped with a rubber policeman in PBS at 4°C and resuspended with 1 ml of homogenization buffer (250 mM sucrose, 8 mM imidazole, pH 7.4). The cells were disrupted by multiple passages through a tuberculin syringe with a 25 gauge needle. The homogenates were then centrifuged at 1000×*g* for 2 min to remove nuclei and unbroken cells. The unbroken cells were resuspended in homogenization buffer and the homogenization cycle was repeated twice. Phagosomes and dense body organelles were pelleted from the combined post-nuclear supernatant by centrifugation at

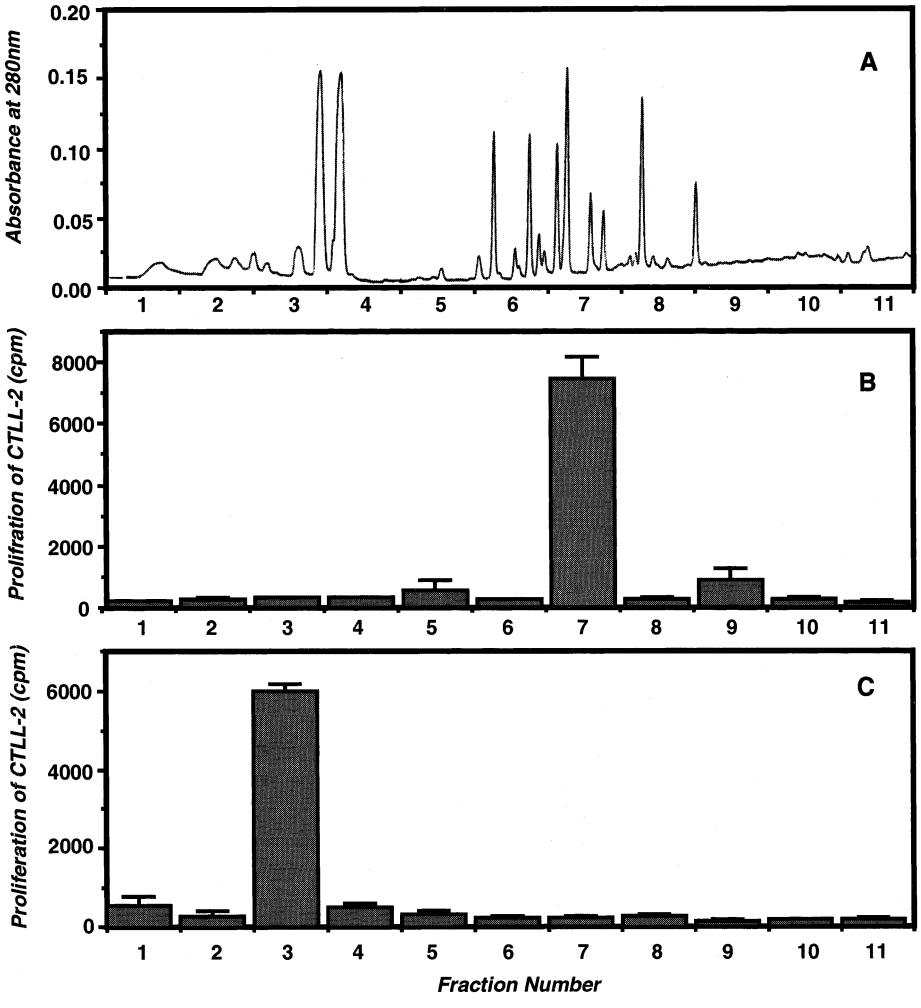

Fig. 1. Detection of OVA-derived MHC class I-restricted antigenic peptides ($OVA_{257-264}$; SIINFEKL) and MHC class II-restricted antigenic peptides ($OVA_{323-339}$) in phagosomes. A murine macrophage cell line (J774.1) was cultured with OVA-coupled latex beads for 15 min. Phagosome fractions were then isolated and the resulting peptides were recovered by sonication and centrifugation as described in Section 2. The peptides fractions were then separated on reverse phase HPLC (A), and each peptide fraction was assayed for the content of either $OVA_{257-264}$ (SIINFEKL) (B) or $OVA_{323-339}$ (C) as described in Section 2. The results are representative of two independent experiments.

40 000×*g* for 15 min. The pellet was resuspended with 0.75 ml homogenization buffer and brought to 40% sucrose by adding the same volume of a 62% sucrose solution. This 40% sucrose supernatant was loaded onto the top of a 0.5 ml cushion of 62% sucrose, after which 1 ml of 35% sucrose, 1 ml of 25% sucrose and 1 ml of 10% sucrose solutions were added. Centrifugation was done in a swinging bucket rotor (Beckman SW55Ti rotor) at 100 000×*g* for 1 h at 4°C. The LBF (latex bead fraction) band was collected from the interface of the 10 and 25% sucrose solutions and resuspended with 12 ml of cold PBS. The LBF was finally pelleted by centrifugation at 40 000×*g* for 15 min at 4°C. In some experiments, the remaining homogenates were then centrifuged at 100 000×*g* for 1 h, and the obtained supernatants were used as cytosol fractions.

2.4. HPLC fractionation of peptides

After the addition of mixtures of protease inhibitors, which consisted of phenyl-methylsulfonyl fluoride (1 mM), pepstatin A (10 μg/ml), leupeptin (10 μg/ml) and E-64-d (10 μg/ml), both phagosome and cytosol fractions were sonicated and then centrifuged at 10 000×*g* for 5 min to remove the latex beads. The supernatant was applied onto a Superdex peptide gel filtration column (0.7×30 cm) which had been equilibrated with PBS. Fractions containing antigen peptides were separated on a reverse phase HPLC column (TSK-80TM, Tisoh, Tokyo). The peptides were eluted with a linear gradient of 0–40% acetonitrile in 0.1% trifluoroacetic acid.

2.5. Antigen presentation assay

HPLC fractions which contained peptides were lyophilized and stored until use. These samples were reconstituted with 300 μl of assay culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, non-essential amino acids, glutamine, sodium pyruvate and antibiotics). For the detection of the $OVA_{257-264}$ (SIINFEKL) in the peptide fractions, 5×10^4 CD8OVA1.3 cells (100 μl) were cultured in triplicate with 100 μl of the reconstituted peptide solution in a flat bottom 96 wells culture plate for 24 h at 37°C in an atmosphere of 5% CO_2. For the detection of the $OVA_{323-339}$, 1×10^4 DOT.1 cells (100 μl) were cultured in triplicate with 100 μl of the reconstituted peptide solution and irradiated (3000 R) BALB/c spleen cells (5×10^4) in a round bottom 96 wells culture plate for 24 h at 37°C in an atmosphere of 5% CO_2. Culture supernatants were assayed for their IL-2 contents by the bioassay using CTLL-2 as previously described [16].

3. Results

3.1. Detection of OVA-derived MHC class I- and class II-restricted antigenic peptides in phagosome

Phagosomes were isolated from J774.1 cells which had internalized OVA-coated latex beads for 15 min. Fractions containing antigen peptides were separated on a reverse phase HPLC column (Fig. 1A). Each peptide fraction was analyzed for the content of either the MHC class I-restricted peptide, $OVA_{257-264}$ (SIINFEKL), or the MHC class II-restricted peptide, $OVA_{323-339}$, by utilizing a SIINFEKL-specific, H-$2K^b$-restricted T cell hybridoma (CD8OVA1.3) or an $OVA_{323-339}$-specific, I-A^d-restricted T cell clone (DOT.1), respectively (see Section 2). As shown in Fig. 1, the MHC class I-restricted peptide (SIINFEKL) and the MHC class II-restricted peptide ($OVA_{323-339}$) were found in fraction 7 (Fig. 1B) and fraction 3 (Fig. 1C), respectively. When the synthesized SIINFEKL and $OVA_{323-339}$ peptides were applied to the same HPLC column, they were eluted in fractions 7 and 3, respectively (data not shown). We therefore conclude that, in the case of phagosomes of J774.1, OVA was degraded into peptides which are immediately ready to bind to the appropriate MHC molecules, i.e. class I or class II. These results indicate that the phagosome per se is capable of digesting antigenic protein molecules into the final form of antigenic peptides. This fact is important particularly when we consider the MHC class I-restricted antigen presentation pathway, an issue which is discussed below.

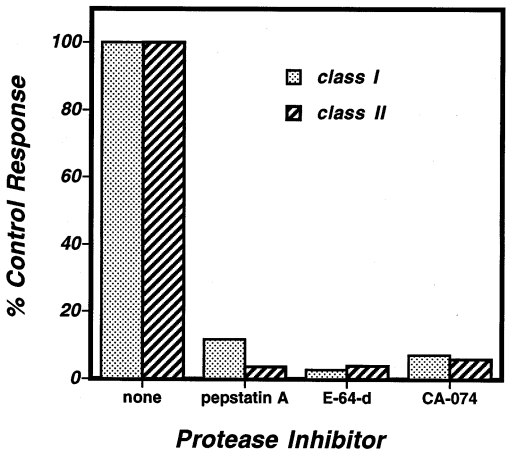

Fig. 2. Effect of protease inhibitors on the generation of OVA-derived MHC class I- and class II-restricted antigenic peptides in phagosomes. J774.1 cells were preincubated with or without 20 μg/ml of pepstatin A, E-64-d or CA-074 for 30 min. The cells were then pulsed with OVA-coupled latex beads for 15 min. Various protease inhibitors were added to the culture medium throughout the incubation. Peptide fractions were isolated from the phagosomes and then separated on HPLC as described in Section 2. The peptide fraction corresponding to either $OVA_{257-264}$ (dotted column) or $OVA_{323-339}$ (hatched column) was assayed for the amount of respective peptide as described in Fig. 1. Data are shown as % of control responses, and the results are representative of four independent experiments.

3.2. Effect of protease inhibitors on the generation of OVA-derived MHC class I- and class II-restricted antigenic peptides in phagosome

We next attempted to determine what types of enzymes are involved in the degradation of OVA antigen into the antigenic peptides in the phagosomes. Various protease inhibitors, including pepstatin A, E-64-d and CA-074, were added to the culture of J774.1 during the phagocytosis of the OVA-coupled latex beads. Peptides which were isolated from the phagosomes were separated on HPLC and fractions corresponding to SIINFEKL or $OVA_{323-339}$ were isolated. Each fraction was assayed for peptide contents as described in Fig. 1. All of the protease inhibitors used in the experiments were found to substantially inhibit the generation of both MHC class I- and class II-restricted antigenic peptides (Fig. 2). It is noteworthy, in this respect, that CA-074, a specific inhibitor of cathepsin B [17], interferes with the generation of both antigenic peptides, indicating that cathepsin B is indeed involved in the degradation of the OVA protein in the phagosome.

3.3. Transfer of the MHC class I-restricted peptide, SIINFEKL, from phagosome to cytosol

It has recently been demonstrated that phagosomes constitute fully competent antigen processing compartments for the formation of peptide–class II MHC complexes [14]. It would be of interest to determine the fate of the MHC class I-restricted peptides, once they were generated in the phagosomes. The question arises as to whether they form peptide–class I MHC complexes in the phagosome. Are they transferred to the cytosol instead as would be expected by the proposed MHC class I-restricted antigen presenting pathway, in which

the peptides are thought to be transported into the ER by the aid of TAP. This issue was approached via kinetic studies. Phagosome fractions were prepared 10, 30 and 60 min after the addition of OVA-coupled latex beads to J774.1 cells. Each fraction was then analyzed for the content of the MHC class I-restricted peptide, SIINFEKL, and the MHC class II-restricted peptide, $OVA_{323-339}$ (Fig. 3A). Both MHC class I- and class II-restricted peptides were barely detectable in the phagosomes after 10 min of culture. It is reasonable that the generated MHC class II-restricted peptide, $OVA_{323-339}$, rapidly associated with MHC class II molecules (I-A^d) after the degradation of OVA in the phagosomes and, as a result, were undetectable in the phagosomes as free peptides. This scenario, however, is not applicable for the MHC class I-restricted peptide, SIINFEKL, since this peptide is H-$2K^b$-restricted and therefore unable to associate with class I molecules (H-$2K^d$, H-$2D^d$) in the J774.1 (H-2^d) phagosomes. The disappearance of SIINFEKL from the phagosomes is consistent with the transfer of the peptide from phagosome to cytosol. Alternatively, the generated peptides were further degraded in the phagosomes by proteases. These possibilities were examined in the following experiment.

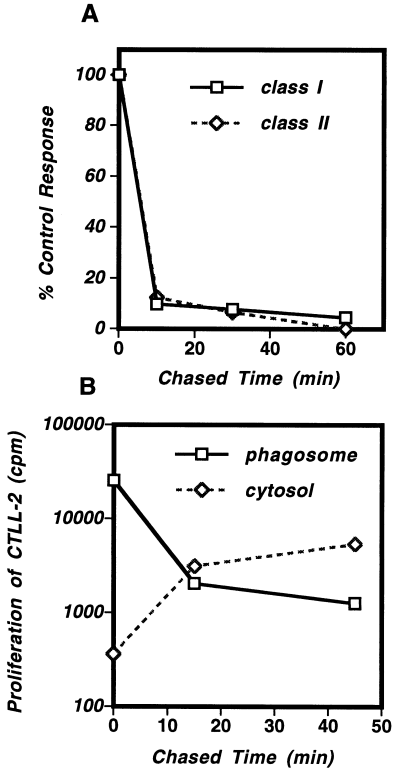

Fig. 3. Transfer of the OVA-derived, MHC class I-restricted peptide (SIINFEKL) from the phagosome to the cytosol. A: Phagosome fractions were prepared 10, 30 and 60 min after the addition of OVA-coupled latex beads to J774.1 cells. The peptide fraction corresponding to either $OVA_{257-264}$ (square symbol, solid line) or $OVA_{323-339}$ (diamond symbol, dotted line) was assayed for the amount of respective peptide as described in Fig. 1. Data are shown as % of control responses, and the results are representative of three independent experiments. B: Both phagosome fraction and cytosol fraction (see Section 2) were prepared 15 and 45 min after the addition of OVA-coupled latex beads to J774.1 cells. The peptide fractions corresponding to $OVA_{257-264}$ were collected from either phagosome fraction (square symbol, solid line) or cytosol fraction (diamond symbol, dotted line) and assayed for the peptide contents as described above. The results are representative of two independent experiments.

The amount of SIINFEKL in either the phagosomes or the cytosol fraction was determined during the chase period (Fig. 3B). Consistent with the results in Fig. 3A, the amount of SIINFEKL in the phagosome was rapidly decreased. In the cytosol fraction, however, the amount of SIINFEKL was gradually increased (Fig. 3B). This inverse correlation between the amounts of SIINFEKL in phagosome and cytosol fractions strongly suggests that the MHC class I-restricted short peptide, SIINFEKL, is in fact transferred from phagosome to cytosol, although we cannot formally exclude the possibility that the SINNFEKL, generated in the phagosome, was further degraded by proteases to some extent.

4. Discussion

In this study, we describe the purification of phagosomes using a discontinuous sucrose gradient. This simple technique was employed to isolate pure preparations of phagosomes from J774.1 macrophages [15]. Since sucrose gradients have been reported to be unsatisfactory due to deleterious effects of the sucrose on the subsequent T cell assay step [14], we isolated peptides from the phagosome fractions by utilizing a reverse phase HPLC fractionation. In order to directly detect MHC-restricted antigenic peptides in murine phagosomes, either an antigen-specific MHC-restricted T clone or T hybridoma cells were utilized. The data demonstrated that an exogenously added OVA protein, which had been conjugated with the latex beads, was phagocytosed by a murine macrophage cell line, J774.1, and degraded into at least two representative MHC class I- and class II-restricted peptides by the action of various cathepsins, including cathepsin B, in the phagosomes. It has not been formally demonstrated that both MHC class I- and class II-restricted antigenic peptides are simultaneously generated in phagosomes. Our observation clearly demonstrates that this is the case, at least for the OVA antigen in murine phagosomes (Fig. 1).

It is possible that a difference in the sensitivity of an assay system could account for the difference in the amount of antigenic peptides detected in the assay, and, because of this possibility, we attempted to minimize the difference in the sensitivities of the two assay systems. In our experiments, nearly identical titration curves (peptide concentrations vs. proliferative responses of CTLL-2) were obtained (data not shown), indicating that nearly the same amount of each peptide was generated in the phagosome (Fig. 1). Moreover, from the results in Fig. 2, it seems that there is little, if any, difference in the usage of cathepsins between the two degradation steps. These results also agree with the notion that two peptides, with distinct MHC preferences, are generated in the same compartment, i.e. the phagosome. We cannot, however, exclude the possibility that there may be a difference in exopeptidases that are involved in the fine trimming of MHC class I- or class II-restricted peptides.

A representative MHC class I (H-$2K^b$)-restricted antigenic peptide, SIINFEKL, is generated in the phagosome of J774.1 cells. Although the haplotype of J774.1 cells is H-2^d, we believe that they are able to generate H-$2K^b$-restricted antigenic peptides, since antigen degradation was largely mediated by cathepsins which apparently have no haplotype preferences. In other words, it is unlikely that there are any differences in

the potential for degradation among phagosomes with various haplotypes. In order to examine whether any MHC class I-restricted antigenic peptide exits from the phagosomes to the cytosol, our assay system is applicable because the generated SIINFEKL is unable to bind to MHC class I molecules in phagosomes, where the appropriate class I molecules, i.e. H-2K^b, are not available. One can argue that the SIINFEKL detected in the cytosol simply resulted from the destruction of phagosomes rather than the active transfer of peptides. We attempted to exclude this possibility by analyzing the amount of phagosomal enzymes in the cytosol preparation. The results of Western blotting analyses indicated that there is no evidence for an increase in the levels of cathepsin D, which is abundant in phagosomes (D. Muno, unpublished observation), in the cytosol during the chase period (data not shown). Collectively, our results strongly suggest that the transfer of antigenic peptides from the phagosomes to the cytosol is a reality and, in fact, occurs (Fig. 3B).

It has already been demonstrated that solid particle-loaded cells show a cytosolic delivery of OVA, indicating that the phagosome to cytosol pathway for OVA is a general and efficient phenomenon in macrophages [10,11,18]. In the present study, we demonstrate the transfer of OVA-derived antigenic peptides from the phagosome to the cytosol. Our observation is of interest when the role of the proteasome in the MHC class I-restricted exogenous antigen presentation pathway is considered. The issue of whether exogenously added protein antigens follow the same pathway as endogenous antigens, which are degraded by the proteasome in cytosol, or they encounter the MHC class I molecules (either from the plasma membrane or newly synthesized in the ER) that enter the phagosomes (cytosolic pathway vs. non-cytosolic pathway; see Section 1) is unclear, at present. Our results may permit a new model to be proposed, which is between the two pathways. However, it is theoretically impossible for the present experimental system to identify the precise location for where the peptide (SIINFEKL), which is transferred to the cytosol, encounters the appropriate MHC class I molecules. A recent study demonstrated that MHC class I molecules can present epitopes of the measles virus F protein in a TAP-independent, NH_4Cl-sensitive manner, suggesting that a small fraction of MHC class I molecules enters MIIC (MHC class II compartment) [19]. There may be a variety of pathways for delivering antigenic peptides to MHC class I molecules. At this moment, the issue of which pathway is most efficient for MHC class I loading of antigenic peptides remains unknown. Transfer of peptides from the phagosome to the cytosol may be one of the most straightforward strategies that is available for the phagocytic APC. The present study may facilitate our attempts to develop new types of vaccines which can deliver antigenic peptides, which can be recognized by CD8$^+$ effector cells, e.g. CTL, via phagocytosis.

In conclusion, the results herein demonstrate that murine phagosomes are capable of simultaneously generating both MHC class I- and class II-restricted antigenic peptides. Evidence for the transfer of small antigenic peptides from the phagosome to the cytosol was also presented. Utilizing phagosomes for the generation as well as the delivery of antigenic peptides for both MHC class I and class II antigen presentation pathway represents one of the most promising approaches to vaccine delivery.

Acknowledgements: We thank Dr. Shimizu for DO11.10 TcR transgenic mice, Dr. Hanada for E-64-d and CA-074, and Dr. Harding for CD8OVA1.3 T hybridoma cells.

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