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Two T Cell Epitopes from the M5 Protein of Viable *Streptococcus pyogenes* Engage Different Pathways of Bacterial Antigen Processing in Mouse Macrophages[1]

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We studied the mechanisms of MHC class II-restricted bacterial Ag processing of the surface fibrillar M5 protein from viable *Streptococcus pyogenes* in murine macrophages. Two previously defined T cell epitopes were studied using T cell hybridomas specific for 308–319/A^d, associated with the cell wall on the surface of streptococci, and 17–31/E^d, located at the protruding amino terminus of M5. Studies with metabolic inhibitors showed that slow (1 h) processing of M5 308–319 occurred in late endosomes and was dependent on newly synthesized MHC class II molecules and microtubules and on communications between early and late endosomes, consistent with engagement of the classical MHC class II processing pathway. In contrast, fast (15 min) bacterial Ag processing of 17–31 occurred in early endosomes independently of newly synthesized MHC class II molecules and microtubules and of trafficking between early and late endosomes, consistent with the recycling MHC class II processing pathway. Finally, bacterial Ag processing of the epitopes exhibited differential sensitivity to blocking with anti-MHC class II Abs. Thus, two T cell epitopes of a single protective Ag from the surface of whole bacteria are routed to distinct MHC class II processing pathways. ***The Journal of Immunology,*** **1998, 160: 5267–5272.**

Efficient processing of bacterial Ags by MHC class II (MHC-II)[3] molecule-expressing professional APC for presentation to CD4$^+$ T cells is one of the milestones in the development of the acquired immunity to infection. The current perception of Ag processing evolved from studies performed on soluble proteins (1–7) and on recombinant Ags expressed in different bacterial species (1, 8–10), as well as on intact bacteria (11–15). Ag uptake and processing is described as engaging many subcellular compartments and pathways, including different cell surface receptors, F-actin microfilament and microtubule cytoskeleton motility motors, endocytic and secretory pathways, and a variety of signaling pathways (16, 17). It is envisaged that internalization of Ags triggers a series of intracellular events, which result in generation and release of immunologically active peptides into late endosomes, which then traffic to distinct compartments for binding to newly synthesized MHC-II molecules. Peptide loading of MHC-II in this so-called classical MHC-II pathway occurs in specialized MHC-II molecule-loading compartment(s) (MIIC) in which the invariant (Ii) chain is degraded by enzymes such as cathepsin S, and DM/H2-M molecules catalyze the exchange of the Ii-derived class II-associated invariant chain (CLIP) peptide for processed antigenic peptides (18–20). Alternatively, Ags may be targeted to early endosomal compartments for binding to MHC-II recycled from the cell surface by the recently described Ii-independent recycling MHC-II pathway, as described for human and mouse B cells, mouse macrophages, and human immature dendritic cells (20–25). Whichever pathway is used, peptide-MHC-II complexes are sorted to the cell surface for subsequent recognition by the TCR complex, leading to T cell activation and cytokine secretion (12, 26). Although the main mechanisms of Ag presentation are now established for several model proteins (22, 27), it is not clear how complex protective bacterial Ags are processed and what determines the route a particular epitope will take following bacterial uptake.

In the present report, we used two CD4$^+$ T cell hybridomas specific for two distinct T cell epitopes of the surface fibrillar M5 protein of *Streptococcus pyogenes*, which is the major virulence factor and protective Ag of group A streptococci (28), to study the mechanisms of bacterial Ag processing in murine macrophages. We have previously shown that both epitopes are processed from viable streptococci for MHC-II-restricted presentation to specific CD4$^+$ T cell clones and T cell hybridomas (29–32). Evidence is presented here that presentation of distinct N-terminal and C-terminal epitopes is segregated into recycling and classical MHC-II processing pathways, respectively.

Materials and Methods

Culture media and chemicals

All culture media ingredients, chemicals, and inhibitors were purchased from Sigma Chemical (Dorset, U.K.) except for tyrphostin A25 (Calbiochem-Novabiochem, Nottingham, U.K.). All of the inhibitors, solvents, and concentrations studied are shown in Table I. All cells were grown in RPMI 1640 medium supplemented with 3.0 mM L-glutamine, 0.05 mM 2-ME, and 10% FBS (v/v).

Cells

The murine macrophage-like cell line J774A.1 (H-2^d, ATCC TIB67; American Type Culture Collection (ATCC), Bethesda, MD) was used in all experiments. T cell hybridomas (HX17 and HY2) were developed by polyethylene glycol (PEG) fusion of two M5 protein-specific T cell clones (X17 and Y2) with BW5147 (TCR$\alpha^-\beta^-$) cells (a gift from Dr. P Marrack, Departments of Microbiology and Immunology and Medicine, University

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[3] Abbreviations used in this paper: MHC-II, class II MHC; MIIC, major histocompatibility class II loading compartment; ER, endoplasmic reticulum; Ii, invariant chain; rM5, recombinant streptococcal type 5 M protein; AlF_4^-, aluminium fluoride anion.

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Table I. *Inhibitors used in this study*

No.	Inhibitor	Solvent	Dose Range Studied	Reference No.
1	AlF_4^- anions[a]	H_2O	6.2 pM–0.5 mM	(37)
2	Brefeldin A	Ethanol	0.1 nM–0.1 mM	(40)
3	Cycloheximide	DMSO	0.1 μM–1.0 mM	(35)
4	Tyrphostin A25	DMSO	10.0 nM–3.0 mM	(38)
5	Vinblastine	DMSO	0.1 nM–0.1 mM	(41)

[a] Potassium fluoride (KF) was used at the constant concentration of 10.0 mM, while $AlNH_4(SO_4)$ varied in the dose range specified, as described in Reference 37. See the relevant sections of the text for descriptions of the mechanisms underlying the blocking effects of the inhibitors.

of Colorado Health Sciences Center, Denver, CO). The expression of the TCR αβ-chains on the T cell hybridomas was confirmed by flow cytometry (data not shown). The specificity of T cell hybridomas HX17 and HY2 for epitopes 17–31/E^d and 308–319/A^d of group A streptococcal type 5 M protein, respectively, was reported previously (32).

The Manfredo strain of *S. pyogenes*, obtained from Dr. Michael A. Kehoe, Department of Microbiology, Newcastle University, Newcastle upon Tyne, U.K. was grown overnight in RPMI 1640 with 10% FBS, and the concentration was adjusted spectrophotometrically to 3×10^8/ml ($A_{600} = 0.6$).

Recombinant M5 protein (rM5) and synthetic peptides

The cloning and expression of rM5 from type 5 *S. pyogenes* strain Manfredo in *Escherichia coli* LE392 and the sequence of the cloned gene have been described previously (29, 33). The following synthetic peptides, covering two T cell epitopes on the M5 protein of *S. pyogenes*, were purchased from the Molecular Biology Facility, Newcastle University, Newcastle upon Tyne, U.K.: 1) 15–33 peptide containing epitope 17–31/E^d; and 2) 300–319 peptide covering epitope 308–319/A^d (29). Peptides were used as HPLC-purified preparations, and purity was confirmed by mass spectrometry.

Ag processing and presentation assay

J774A.1 macrophages (6×10^4/well) were allowed to adhere to the bottom of 48-well plates (Bibby Sterilin, Staffordshire, U.K.) for 1 h; the cells were pretreated with inhibitors for 30 min, except if stated otherwise; and viable streptococci (3×10^6/well) or rM5 (1.0 μg/ml) were added. After 1 h of incubation at 37°C in a humidified CO_2 incubator, nonphagocytozed bacteria were killed with gentamicin (50 μg/ml), and the plates were incubated for an additional 3 h. The macrophages were fixed with 1.0% paraformaldehyde for 10 min, the reaction was stopped with ice-cold 0.06% Gly-Gly (34), and the wells were washed with PBS to remove the extracellular bacteria, the fixative, and any remaining inhibitors. T cell hybridoma cells were added (3×10^4/well), the plates were incubated at 37°C in a CO_2 incubator for 24 h, and the culture supernatants were collected and frozen at −20°C overnight before the IL-2 assay. In some experiments, J774A.1 cells were pretreated with anti-MHC-II mAb M5/114.15.2 (anti-$A\beta^{b,d,q}$; anti-$E^{d,k}$), used as ammonium sulfate precipitate of culture supernatant from hybridoma TIB120 obtained from ATCC, for 30 min before challenging with Ag. After a 4-h incubation, macrophages were either fixed or not before adding T cell hybridomas.

Particular attention was paid to ensure that any inhibition observed did not result from the nonspecific activity of inhibitors. Synthetic peptide controls were performed for each dilution of the inhibitor, and the viability of J774A.1 cells before fixation was confirmed in all experiments. The following controls were routinely used: 1) responses in the absence of inhibitors, 2) background responses in the absence of Ag, and 3) presentation of the relevant peptide (4.0 μg/ml). The optimal concentrations of the fixative, inhibitors, and the cells involved, as well as the timing, were determined in separate experiments. All experiments were repeated at least three times, and the data for a representative experiment are shown.

IL-2 assay

Culture supernatants (diluted 1:2) obtained from Ag processing assays were screened for IL-2 content, measured as the proliferative response of CTLL-2 cells (10^4/well). The assays were performed in flat-bottom 96-well Microtiter plates (Becton Dickinson, Cowley, Oxford, U.K.) on two separate occasions in duplicates for 24 h at 37°C in a humidified CO_2 incubator. The cultures were pulse labeled overnight with 0.4 μCi of [^{3}H]thymidine (TRA310, sp. act., 2.0 Ci/mmol; Amersham International, Buckinghamshire, U.K.) and harvested on glass fiber membranes, and radioactivity was quantitated using a direct beta counter (Matrix 9600, Packard Instrument, Meriden, CT).

Statistics

Descriptive statistics including calculations of the mean and the SE of the mean (SEM) were performed at $p = 0.05$ on an IBM-compatible PC using MicroSoft Excel 5.0 and Prism 2.0 software (GraphPad, San Diego, CA).

Results

Two T cell epitopes from streptococcal M5 protein are segregated to distinct MHC-II pathways for Ag processing

The classical and recycling MHC-II processing pathways use different pools of MHC-II molecules, and thus can be distinguished according to their susceptibility to brefeldin A, which disrupts the Golgi network, and cycloheximide, which inhibits protein synthesis. Both inhibitors block the classical MHC-II processing pathway by preventing accumulation of newly synthesized MHC-II molecules in peptide-loading compartments (18, 20, 23, 35).

Brefeldin A inhibited processing of 308–319 from viable streptococci, but had no apparent effect on the processing of 17–31 (Fig. 1*A*). Similarly, pretreatment of J774A.1 macrophages with cycloheximide for 2 h eliminated presentation of 308–319 and only partially blocked the processing of 17–31 from viable bacteria (Fig. 1*B*). The data support the interpretation that processing of viable streptococci for 308–319 presentation followed the classical MHC-II pathway using newly synthesized MHC-II molecules and requiring functional endoplasmic reticulum (ER)-Golgi transport. In contrast, the data indicate that 17–31 was presented by the recycling MHC-II molecules independent of ER-Golgi communications.

We also studied processing of soluble rM5 protein, which followed largely the same pattern as for viable bacteria, except that 17–31 presentation was blocked by cycloheximide and brefeldin A treatment for 2 h (data not shown), suggesting that ER-Golgi transport of some newly synthesized proteins is required for the recycling MHC-II pathway. Finally, synthetic peptide presentation was unaffected by either inhibitor.

Ag processing of two T cell epitopes involves different endocytic compartments

There is recent evidence suggesting that peptide loading can occur both in late endosomal and early endosomal compartments, which require newly synthesized or recycling MHC-II molecules, respectively (18, 20, 21, 27). Membrane transport from early to late endosomes has been shown to be dependent on microtubules and is regulated by protein kinases, GTP-binding proteins, and other proteins (17). Thus, we used metabolic inhibitors to distinguish between early and late endosomes as the main Ag processing compartments for viable streptococci.

Vinblastine disrupts the microtubule network, which is essential for translocation of endocytosed Ags between early and late endosomes (36). Presentation of 17–31 from viable streptococci was not blocked by vinblastine (Fig. 2*A*), pointing to early endosomes as the main Ag-processing compartment for this epitope. In contrast, presentation of 308–319 from bacteria was consistently more dependent on functional microtubules, suggesting that Ag processing of this epitope occurred mainly in late endosomal/lysosomal compartments.

Another mechanism regulating communication between early and late endosomes involves GTP-binding proteins (17). This step has been shown to be sensitive to aluminium fluoride anions (AlF_4^-), which have structural similarity to the γ-phosphate of GTP and therefore bind and inactivate the GDP-bound form of trimeric G proteins (37). AlF_4^- profoundly blocked processing of

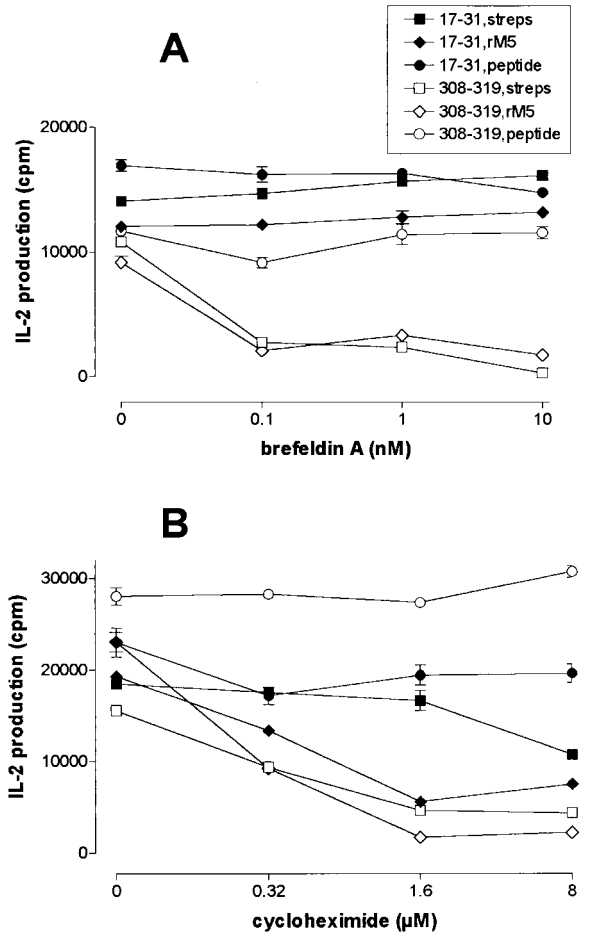

FIGURE 1. Effect of brefeldin A and cycloheximide on presentation of two M5 protein T cell epitopes. J774A.1 macrophages were pretreated with different concentrations of brefeldin A for 15 min (*A*) or with cycloheximide for 2 h (*B*) before challenge with viable streptococci (3×10^6/well; squares), rM5 (1.0 μg/ml; diamonds), or synthetic peptides (4.0 μg/ml; circles). After a 4-h pulse with Ag, cells were fixed and T cell hybridomas added: HX17 specific for 17–31 (closed symbols), HY2 specific for 308–319 (open symbols). T cell hybridoma responses were measured as CTLL proliferation in cpm (± SEM) as shown on the *y*-axis. The experiments were performed on at least three occasions, and data from a representative experiment is shown.

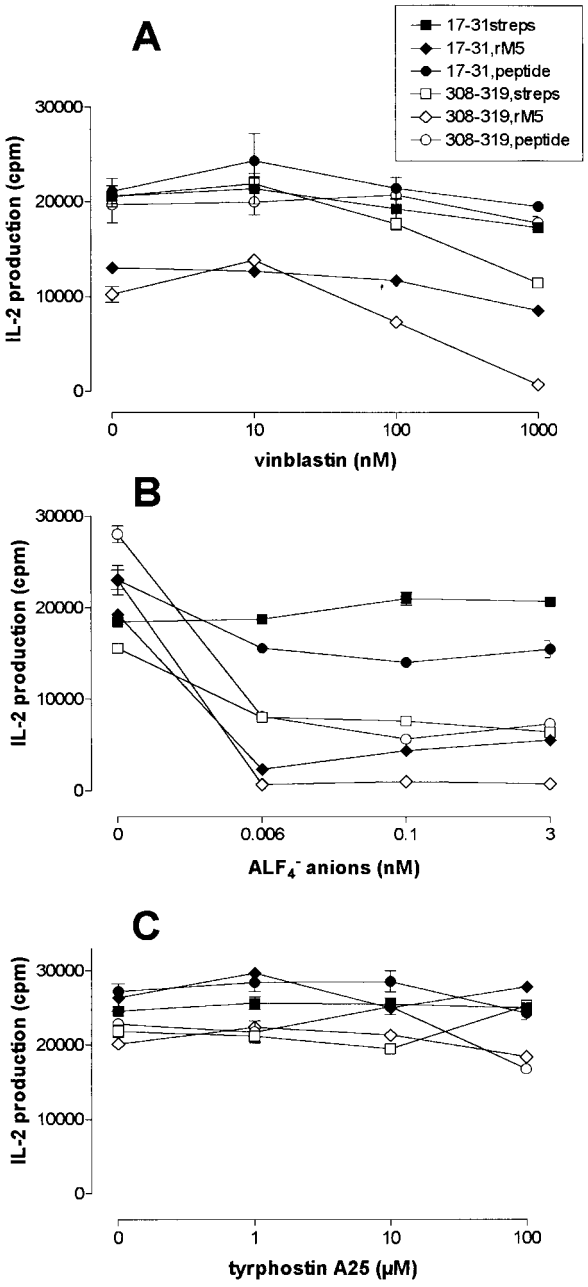

FIGURE 2. Different endosomal compartments participate in processing of two M5 protein-specific T cell epitopes. Presentation of T cell epitopes 17–31 and 308–319 from viable streptococci (squares), rM5 (diamonds), or synthetic peptides (circles) to specific T cell hybridomas: HX17 (closed symbols) and HY2 (open symbols). Different mechanisms controlling communication between early and late endosomes/lysosomes were inhibited by vinblastine (*A*), AlF_4^- (*B*), and tyrphostin A25 (*C*) (see text). For other details, see the legend to Figure 1.

308–319 from bacteria, implicating GTP-binding protein-dependent endosomal transport in the processing of this epitope (Fig. 2*B*). In contrast, AlF_4^- did not block processing of 17–31 from viable streptococci (Fig. 2*B*). These data also suggest that processing of streptococci for presentation of 17–31 occurred in early endosomes.

To distinguish between late endosomes and lysosomes as the intracellular compartments used for processing of 308–319, we used tyrphostin A25, which blocks protein tyrosine kinase-dependent regulation of endosomal transport between late endosomes and lysosomes (38). Tyrphostin A25 did not reduce the level of presentation of 308–319 (or M5 17–31), indicating that bacterial processing of 308–319 was confined to the late endosomal, rather than the lysosomal, compartment (Fig. 2*C*).

Processing of both epitopes from soluble rM5 resembled that observed for viable bacteria, except that 17–31 presentation was inhibited by AlF_4^- anions, implying involvement of GTP-binding proteins. Presentation of synthetic peptides was unaffected by these inhibitors, except that 308–319 was blocked by AlF_4^- anions.

Different kinetics of Ag presentation of two M5 T cell epitopes

Classical and recycling MHC-II pathways can be distinguished by the kinetics of Ag presentation (23, 25). Figure 3 shows that presentation of 17–31 was detected from 15 min after challenge with streptococci, reaching a plateau by 60 min, consistent with processing via the recycling MHC-II pathway. In contrast, 308–319

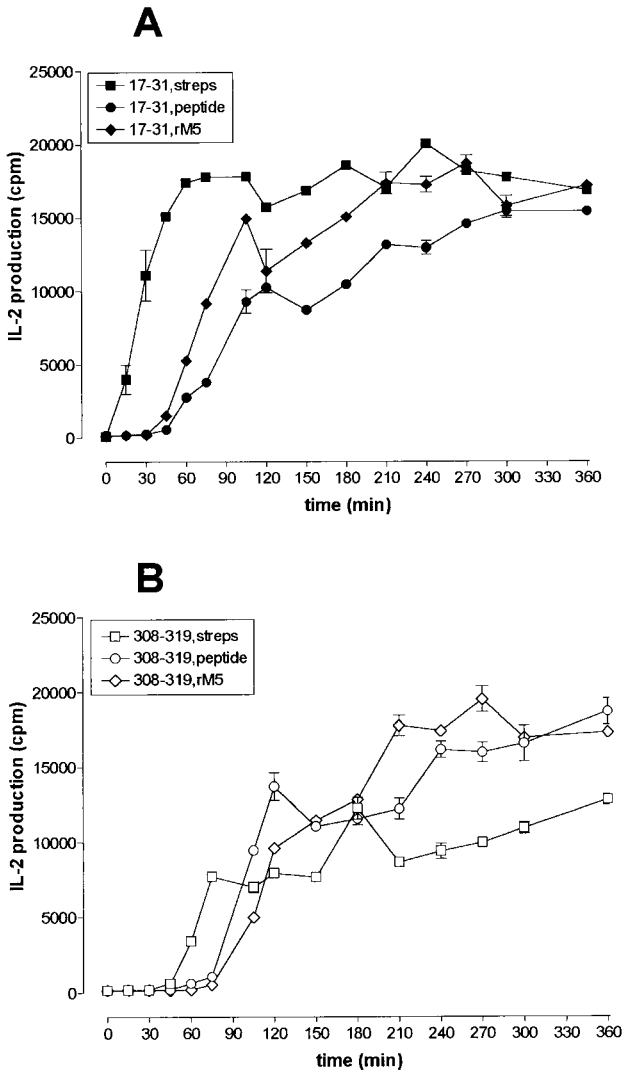

FIGURE 3. Kinetics of Ag processing of two M5 protein-specific T cell epitopes from viable streptococci. Presentation of 17–31 (*A*; filled symbols) or 308–319 (*B*; open symbols) was studied as a function of time between the challenge with viable streptococci (squares), rM5 (diamonds), or synthetic peptides (circles) and fixation of Ag processing. For other details, see the legend to Figure 1.

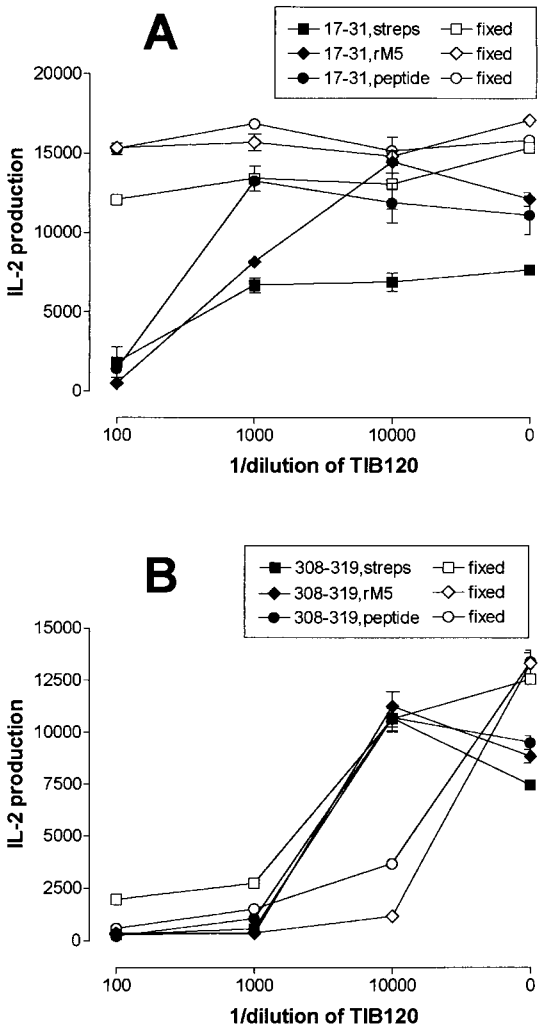

FIGURE 4. The effect of anti-MHC class II mAbs (TIB120; anti-$A\beta^{b,d,q}$; anti-$E^{d,k}$) on presentation of two streptococcal M5 protein-specific T cell epitopes. After processing of viable streptococci (squares), soluble rM5 (diamonds), or synthetic peptides (circles), mouse macrophages were either fixed (open symbols) or not (filled symbols) before adding T cell hybridomas specific for 17–31 (*A*) or 308–319 (*B*). Error bars denote SEM.

was processed more slowly and was detectable from 60 min, with the level increasing up to 360 min, consistent with processing via the classical MHC-II pathway. Presentation of both epitopes from soluble rM5 and synthetic peptides uniformly showed the slower kinetic patterns.

Differential effect of anti-MHC-II mAb on presentation of 17–31 and 308–319 T cell epitopes of the streptococcal M5 protein

Distinct patterns of inhibitory activity of anti-MHC-II mAb TIB120 (specific for $A\beta^{b,d,q}$ and $E^{d,k}$) was observed for presentation of the two epitopes to specific T cells. If J774A.1 cells were not fixed after challenge with viable streptococci, presentation of both epitopes was completely blocked by anti-MHC-II mAb (Fig. 4), with 308–319 being about 10 times more sensitive. However, if J774A.1 were fixed, TIB120 failed to inhibit presentation of 17–31 even at the highest concentrations used (Fig. 4*A*). The data on presentation of soluble rM5 protein and synthetic peptides were consistent with bacterial Ag processing for presentation of both epitopes, suggesting a differential sensitivity of the classical and recycling MHC-II pathways to anti-MHC-II mAb.

Discussion

We have developed a model system to study Ag processing of viable group A streptococci using J774A.1 macrophages as APC and T cell hybridomas specific for two previously defined $CD4^+$ T cell epitopes located near the protruding amino terminus (17–31/E^d) or in the carboxyl-terminal region (308–319/A^d) of the streptococcal surface fibrillar type 5 M protein. In this report we used metabolic inhibitors to demonstrate that processing of the C-terminal epitope 308–319 from viable streptococci was dependent on newly synthesized MHC-II molecules, ER-Golgi transport, and intact communications between early and late endosomes. The results obtained with inhibitors are consistent with the slow (1 h) kinetics of 308–319 presentation observed, together implying the engagement of the classical MHC-II Ag processing pathway. In contrast, presentation of the N-terminal epitope 17–31 was independent of ER-Golgi transport and newly synthesized MHC-II

molecules and commenced as early as 15 min after challenge with viable bacteria, indicating that bacterial processing occurred by an essentially different route, via the recycling MHC-II pathway. Previous studies described the recycling MHC-II pathway operating in mouse and human B cells (20, 21, 23, 24), human immature dendritic cells (23), and mouse macrophages (25) for processing of different model soluble Ags. Our study performed in mouse macrophages suggests that this pathway is used for efficient Ag processing of a protruding N-terminal T cell epitope of the M5 protein on the surface of viable *S. pyogenes*.

Morphologic studies revealed that most of the intracellular MHC-II molecules could be visualized by immunoelectron microscopy in the high density multivesicular or multilaminar endocytic compartments that express markers of late endosomes/lysosomes (MIIC) (18), and in low density endosomes (CIIV), which are devoid of lysosomal markers (39). MIIC are thought to metabolize most Ags for presentation by newly synthesized MHC-II molecules of the classical MHC-II processing pathway (16, 19, 27), while low density endosomes are involved in the presentation of some Ags by MHC-II molecules recycled from the cell surface, constituting the recycling MHC-II processing pathway (20, 21, 23, 24). In the current study, we showed that two T cell epitopes of a single protein on the bacterial surface were clearly routed to distinct Ag processing compartments in the course of bacterial Ag processing. The segregation mechanism of the two epitopes from the same protein for targeting to distinct MHC-II processing pathways is under investigation. Targeting to distinct processing pathways would be expected if an amino-terminal fragment of M5 that contains 17–31 was enzymatically cleaved from the streptococcal surface (or from soluble rM5), separating it from the remaining fragment of M5 containing 308–319, for entry into the early endosomal compartment. However, the differences may also lie in the primary structure of the epitopes and flanking amino acid sequences, which could influence peptide targeting or transport. The dose-response titrations of the two T cell hybridomas are superimposable (data not shown), indicating that the affinity of T cell receptors is unlikely to account for the differences seen. We do not know whether these differences are dependent on the MHC haplotype, as our results are confined to the H-2^d mice. The two epitopes studied bind different MHC-II molecules: 17–31 to E^d and 308–319 to A^d, although other reports have not shown preferential usage of a particular MHC-II molecule for presentation via the recycling vs the classical MHC-II pathway (20, 21, 23–25).

Our study also revealed that bacterial Ag processing of 17–31 and 308–319 exhibited differential sensitivity to blocking with anti-MHC-II mAb, which was particularly pronounced in fixed macrophages. Thus, presentation of 308–319 was completely blocked, whereas presentation of 17–31 was not inhibited by anti-MHC-II mAb, suggesting that classical and recycling MHC-II pathways are differentially blocked by anti-MHC-II Abs. The mechanisms of this differential blocking are under investigation.

Our demonstration that epitopes from the same protective Ag on the surface of an intact bacterial pathogen can be processed by different MHC-II pathways has implications for Ag delivery systems used for the development of vaccines.

Note added in proof. The authors have since published the following related study: Deluig, A. A., and J. H. Robinson. 1998. Different endosomal proteolysis requirements for antigen processing of two T-cell epitopes of the M5 protein from viable *streptococcus pyogenes. J. Biol. Chem. 273:3291.*

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