Cutting edge: neosynthesis is required for the presentation of a T cell epitope from a long-lived viral protein

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Abstract: CTLs recognize peptide epitopes which are proteolytically generated by the proteasome and presented on MHC class I molecules. According to the defective ribosomal product (DRiP) hypothesis, epitopes originate from newly synthesized polypeptides which are degraded shortly after their translation. The DRiP hypothesis would explain how epitopes can be generated from long-lived proteins. We examined whether neosynthesis is required for presentation of the immunodominant epitope NP118 of the lymphocytic choriomeningitis virus nucleoprotein, which has a half-life of >3 days. Two days after nucleoprotein biosynthesis was terminated in a tetracycline-regulated transfectant, the presentation of the NP118 epitope ceased. This indicates that NP118 epitopes are generated from newly synthesized nucleoproteins rather than from the long-lived pool of nucleoproteins in the cell. Therefore, the lymphocytic choriomeningitis virus nucleoprotein is the first substrate for which a major prediction of the DRiP hypothesis, namely the requirement for neosynthesis, is shown to hold true.

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Cutting Edge: Neosynthesis Is Required for the Presentation of a T Cell Epitope from a Long-Lived Viral Protein

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Cell lines
B8 is a BALB/c-derived fibroblast line obtained by SV40 infection in vitro (5). The T cell hybridoma LCMV-NP118 is specific for the NP118 epitope of LCMV (5) and T2-L is an H-2Lb transfectant of the TAP-deficient lymphoblastoid line T2. The cells were grown in IMDM supplemented with 10% FCS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin.

LCMV and infection of cell lines
LCMV-WE strain was grown and titrated on L929 cells as previously described (5). The infection of 10^5 cells in vitro was performed at a multiplicity of infection of 0.05 in RPMI 1640 medium without FCS in a volume of 1 ml under agitation at room temperature for 30 min. Subsequently, the cells were plated in complete growth medium.

Generation of the pTet-SpliceLCMV-NP construct
The LCMV-WE cDNA was cloned into the tetracycline (tet)-regulated expression construct pTet-Splice (6) via EcoRI and SpeI sites, thus yielding the plasmid pTet-SpliceLCMV-NP.

Transfections
B8 cells were cotransfected by calcium phosphate precipitation with the pTet-TAK plasmid encoding the tet-responsive inducer (6) and the pLXSH plasmid encoding a hygromycin resistance gene. Clones were selected with hygromycin B (400 μg/ml) in medium containing 1 μg/ml tet. The presence of pTet-TAK was confirmed by genomic PCR analysis using the oligonucleotides 5'-ATGCTAGATTAGATAAAGTAAG-3' and 5'-CTACCCACCCTACTCTGCAA-3', specific for the coding sequence of the transactivator gene. B8T.A4, a clone which showed high tet expression in the absence of tet but very low expression in the presence of tet, was selected for supertransfections. B8T.A4 cells were transfected with pTet-SpliceLCMV-NP and the pLXSP plasmid encoding a puromycin resistance gene. Puromycin-resistant clones were cultured in IMDM supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 400 μg/ml hygromycin, 5 μg/ml puromycin, and 1 μg/ml tet. The integration of the pTet-SpliceLCMV-NP construct into the genome was confirmed by genomic PCR using oligonucleotides 5'-AGAAAGGAGGAAGGAGGACTATAA-3' and 5'-TGAATTCCTCCTAGCTTGTAG-3'.

Cytolytic assay and hybridoma assay
As target cell we used unpulsed B8 cells or B8 cells loaded with the NP118 peptide RPQASGVYM at 10^{-7} M in PBS for 1 h. Splenocytes from a BALB/c mouse which had been infected 8 days before with 200 pfu of LCMV-WE i.v. were used as effectors in chromium release assays. Loading of target cells with chromium and performance of the cytolytic assay were exactly as previously described (5). The hybridoma/report assay was used with the LCMV-NP118-specific T cell hybridoma as outlined elsewhere (5).

Metabolic labeling and immunoprecipitation
B8 cells were either left uninfected or were infected with LCMV-WE 24 h before metabolic labeling. B8NP64 cells were grown in the presence or absence of 1 μg/ml tet at least 5 days before labeling. Cells were labeled with [35S]methionine/[35S]cysteine for 3 h, chased for indicated time periods, and immunoprecipitated with mAb KL53 as previously described (1).

Northern blot
Total RNA was extracted from cells using TRIZol Reagent (Life Technologies, Basel, Switzerland), separated on agarose formaldehyde gels, transferred to a membrane, and radioactively probed as described elsewhere (7). 32P-Labeled probes were generated on nucleoprotein and hypoxanthine-guanine phosphoribosyltransferase (HPRT) cDNA templates using random primers as well as the specific primers HPRT1 (5'-CAGAGGACTAGAA CACCTGC-3'), HPRT2 (5'-GCGTTGAAAGGACTCT-3'), NP1 (5'-AGAGAAGGAGAAGCCTTATTATAAAT-3'), and NP2 (5'-CCGACAGAC CCAAATTATTATA-3').

Results
The LCMV nucleoprotein is a very long-lived protein in LCMV-infected cells and in a tet-regulated nucleoprotein transfectant
LCMV is a small (–) strand RNA virus which consists of only two structural proteins, the glycoprotein and the nucleoprotein. Previously we observed during pulse chase experiments that the half-life of the nucleoprotein is much longer than that of the glycoprotein (1). To determine the degradation rate of the LCMV nucleoprotein in B8 mouse fibroblast cells, these cells were infected with the WE strain of LCMV, and after 1 day of infection the cells were metabolically labeled and chased over a period of 3 days. Remarkably, no evidence for a degradation of the LCMV nucleoprotein could be obtained within 3 days (Fig. 1A).

To investigate the role of protein neosynthesis for the presentation of the nucleoprotein epitope NP118, we generated a transfectant of B8 cells in which the nucleoprotein is expressed from a tet-regulated promoter. Initially, B8 cells were stably transfected with the expression construct pTet-tTAK, which encodes a tet-responsive transcriptional inducer (6). The transfectants were tested by transient supertransfection of tet-regulated β-galactosidase or luciferase reporter constructs, and a clone designated B8tTA.F4 was chosen for additional experiments. This clone was stably supertransfected with the plasmid pTet-SpliceLCMV-NP, which contains the full-length cDNA of the LCMV-WE nucleoprotein under the control of a tet-repressible promoter (6). Transfectants were prescreened with genomic PCR, and a clone, designated B8tNP64, which presented the NP118 epitope in the absence but not in the presence of tet (Fig. 4A), was selected for further analysis.

The degradation rate of the LCMV nucleoprotein was determined in the B8tNP64 clone in a pulse chase experiment (Fig. 1B). A quantification of the nucleoprotein immunoprecipitates from LCMV-WE-infected B8 cells and the B8tNP64 clone in the absence of tet showed that the amount of nucleoprotein was about eightfold lower in B8tNP64 cells. The expression of the nucleoprotein in B8tNP64 cells is completely suppressed in the presence of tet, as evidenced in the third lane of Fig. 1B. Similar to what we had observed in LCMV-WE-infected B8 cells, the half-life of the nucleoprotein in B8tNP64 cells is longer than 3 days. It should be noted that the pulse chase experiment with B8tNP64 cells was performed such that tet was added to the growth medium during the chase, thus preventing nucleoprotein neosynthesis during the chase period.
Determination of the half-life of nucleoprotein mRNA in tet-regulated transfectants and of H-2L<sup>d</sup>/NP118 complexes on the cell surface

To determine the role of nucleoprotein neosynthesis for NP118 presentation it was important to investigate how long the mRNA for the nucleoprotein would persist in B8tNP64 transfectants after the cells were treated with tet. The Northern blot shown in Fig. 2 illustrates that B8tNP64 cells which had been grown for 5 days in the presence of tet do not express nucleoprotein mRNA (Fig. 2, lane 1). When the clone was grown for 5 days in the absence of tet the nucleoprotein mRNA was readily detected in B8tNP64 cells (Fig. 2, lane 2). The amount of nucleoprotein mRNA in B8tNP64 cells was about eightfold lower than in B8 cells which had been infected with LCMV-WE for 2 days, a result which is in accordance with the quantification of nucleoprotein immunoprecipitates (Fig. 1B) which had been performed with aliquots of the same cells. Only 24 h after the addition of tet to these cells (Fig. 2, lane 3) nucleoprotein mRNA was not detectable anymore, indicating that the tet-mediated ablation of nucleoprotein transcription was rapid and the nucleoprotein mRNA in B8tNP64 cells was degraded within 1 day. Consistently, we could not detect nucleoprotein neosynthesis in metabolic labeling and immunoprecipitation experiments after cells had been treated with tet for 6, 24, 48, 72, or 96 h (data not shown).

Another factor which affects the duration of Ag presentation after shutting off nucleoprotein neosynthesis is the half-life of H-2L<sup>d</sup>/NP118 complexes on the cell surface. We determined by flow cytometry how long the NP118 synthetic nonamer would be able to stabilize H-2L<sup>d</sup> complexes on the surface of T2-L<sup>d</sup> cells. The loss of H-L<sup>d</sup>/NP118 complexes from the cell surface was complete after 16 h of incubation at 37°C (data not shown), indicating that for the stimulation of T cells new supply of epitopes is required after this time period.

Neosynthesis of the LCMV nucleoprotein is required for the presentation of the NP118 epitope

To determine how the tet-mediated ablation of synthesizing nucleoprotein mRNA and, as a consequence, of the nucleoprotein itself would affect NP118 presentation in B8tNP64 cells, we performed lacZ assays based on the activation of the H-2L<sup>d</sup>/NP118-specific T cell hybridoma LCMV-NP118 (5). In a first experiment we tested the specificity of the hybridoma, which reacted with NP118-pulsed B8 cells and with B8tNP64 cells grown in the absence of tet but not with unpulsed B8 cells or B8tNP64 cells grown in the presence of tet (Fig. 3A). Moreover, we established that NP118 presentation on LCMV-infected B8 cells was not affected by tet (data not shown). Next, we monitored NP118 presentation on B8tNP64 cells which had been grown in the absence of tet and were subsequently cultivated for 0, 1, 2, and 3 days in the presence of tet (Fig. 3B). Only 1 day after cultivation with tet, NP118 presentation was reduced by 50%, and on the second day of tet treatment the NP118 presentation was at background levels obtained with uninfected B8 cells. Aliquots of the same B8tNP64 cells used as stimulators in this experiment were used in parallel for the immunoprecipitation and Northern blot analysis shown in Fig. 1B and Fig. 2, respectively. It is therefore evident that nucleoprotein neosynthesis is required to maintain NP118 presentation and that the presentation of this epitope cannot be fueled from the long-lived nucleoprotein molecules, which remained at the same level in B8tNP64 over 3 days. The same conclusion was reached in an independent experiment when NP118 presentation was monitored in a cytolytic assay with LCMV-specific primary CTLs as effectors (Fig. 4). Even with this very sensitive read-out system, a sharp drop in NP118 presentation was detected 1 day after the termination of nucleoprotein synthesis in B8tNP64 cells and no residual NP118 presentation remained on the third day of cultivation in the presence of tet. Taken together, our data clearly demonstrate a requirement for nucleoprotein neosynthesis for the presentation of the immunodominant epitope NP118.

![FIGURE 2. Northern blot analysis of LCMV nucleoprotein mRNA content in LCMV-infected B8 cells and in the tet-regulated nucleoprotein transfectant B8tNP64. Cells were grown in the presence (+tet) or absence (−tet) of tet. In the case of −/− tet samples, B8tNP64 cells had been cultivated without tet and were then grown in the presence of tet for the indicated time periods. Total RNA was blotted and hybridized with 32P-labeled LCMV nucleoprotein and HPRT probes. The small size difference between the viral and vector derived mRNAs is probably due to untranslated regions of different length.](image)

![FIGURE 3. Analysis of NP118 presentation by B8tNP64 cells with a T cell hybridoma-based lacZ assay. Activation of the LCMV-NP118 hybridoma leads to β-galactosidase production, which is measured in a chromogenic assay. A, Comparison of NP118 presentation by untreated or NP118 peptide-loaded B8 cells with B8tNP64 cells grown in the absence or presence of tet. B, NP118 presentation was determined for B8tNP64 cells which had been cultured in the absence of tet and were then grown in the presence of tet for the indicated number of days (−/− tet, filled bars). For each assay day, B8tNP64 cells which had been grown in the absence (−tet) or presence (+tet) of tet were used as controls. The data represent means and SEM of triplicate values.](image)
Discussion

In this work we tried to reconcile two paradoxical experimental findings. On the one hand, the LCMV nucleoprotein contains two immunodominant T cell epitopes which are presented in a proteasome-dependent manner (1). On the other hand, the nucleoprotein is a long-lived protein which is not detectably degraded within a period of 3 days. Two hypotheses have been proposed which could resolve the apparent paradox. The first hypothesis is the peptone hypothesis proposed in 1989 by Boon and Van Pel (8). It suggests that class I ligands may stem from polypeptides (peptons) which were translated from short RNAs generated randomly by promoter- and frame-independent transcriptional events. This hypothesis is inconsistent with our results because NP118 presentation correlates strictly with the activation of the tet-repressible promoter which drives nucleoprotein transcription. The second hypothesis is the DRiP hypothesis put forward in 1996 by Yewdell et al. (2). It states that T cell epitopes are derived from defective ribosomal products which are not translated or folded properly and are degraded shortly after their synthesis. A critical aspect of this hypothesis, namely the requirement of neosynthesis for class I-restricted presentation, is in accordance with our results. Whether some or all of these rapidly degraded nucleoproteins are defective or whether they were translated correctly but simply did not fold fast enough to escape degradation has not been addressed in the present study and remains to be determined.

Although we cannot rule out that a minor contribution to the pool of NP118 epitopes stems from the catabolism of preexisting and long-lived nucleoproteins, this supply is clearly insufficient to be recognized by NP118-specific CTLs. Hence, the NP118-specific CTL response will lyse only those cells which actively synthesize the nucleoprotein, and cells which managed to suppress the transcription and translation of the LCMV nucleoprotein with help of antiviral cytokines like IFN-α and IFN-γ may escape lysis and irreversible destruction through CTLs.

References