INFECTION AND IMMUNITY, Apr. 2004, p. 2412–2415 Vol. 72, No. 4
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.4.2412–2415.2004
Copyright © 2004, American Society for Microbiology. All Rights Reserved.

HLA-A2-Restricted CD8$^+$-Cytotoxic-T-Cell Responses to Novel Epitopes in *Mycobacterium tuberculosis* Superoxide Dismutase, Alanine Dehydrogenase, and Glutamine Synthetase

Yuzhi Dong,[1] Sandra Demaria,[2] Xuming Sun,[1] Fabio R. Santori,[2] Bill M. Jesdale,[3] Anne S. De Groot,[3] William N. Rom,[2] and Yuri Bushkin[1]*

Laboratory of Molecular Immunology, Public Health Research Institute, Newark, New Jersey 07103[1]; New York University School of Medicine, New York, New York 10016[2]; and Brown University School of Medicine, Providence, Rhode Island 02912[3]

Received 21 July 2003/Returned for modification 20 October 2003/Accepted 16 January 2004

Major histocompatibility complex class I-restricted CD8$^+$ cytotoxic T lymphocytes (CTL) are implicated in protective Th1 immunity to *Mycobacterium tuberculosis* infection. We report the identification of three novel HLA-A*0201-restricted CTL epitopes within mycobacterial superoxide dismutase (SodA), L-alanine dehydrogenase (AlaDH), and L-glutamine synthetase (GlnS) proteins.

Cellular immunity is essential for protection against infections caused by intracellular pathogens, including *Mycobacterium tuberculosis* (20). Major histocompatibility complex (MHC) class I-restricted CD8$^+$ cytotoxic T lymphocytes (CTL) contribute to the control of *M. tuberculosis* infection by mediating specific effector functions, including lysis of infected cells, direct antimicrobial activity, and release of Th1 cytokines that activate antimycobacterial mechanisms in macrophages (7, 19, 23). Still, only a small number of MHC class I-restricted CTL epitopes have been identified within a few mycobacterial proteins. The human CD8$^+$ CTL specific for immunodominant epitopes derived from *M. tuberculosis* proteins lyse infected cells and readily release gamma interferon (IFN-γ) upon recognition of their targets (3, 4, 8, 11, 12, 15, 17, 21). Several mycobacterial enzymes including SodA, AlaDH, and GlnS were previously identified in culture fluids of in vitro-growing bacilli (1, 2, 22). These enzymes may have important functions in pathogenicity of mycobacteria. Their extracellular expression reflects intracellular abundance and stability and may result from bacterial autolysis (26). Whether mycobacterial superoxide dismutase (SodA), L-alanine dehydrogenase (AlaDH), and L-glutamine synthetase (GlnS) can induce human CD8$^+$-CTL responses has not been previously investigated.

First, the relative affinities and stabilization capacities of synthetic peptides selected from the *M. tuberculosis* proteins SodA, AlaDH, and GlnS for the presence of HLA-A*0201 allele-binding motifs were measured in stabilization assays with T2 cells (16, 24) (Table 1). Binding of exogenous peptides to surface HLA-A2 molecules increased levels of expression of peptide-ligand complexes in T2 cells, as detected by immunostaining with conformation-specific monoclonal antibody (MAb) MA2.1 and flow cytometry, and allowed for selection of potential CTL epitopes. Next, memory CTL responses against peptide-pulsed T2 target cells were measured in peripheral blood mononuclear cells (PBMC) obtained from HLA-A*0201-positive tuberculosis (TB) patients ($n = 8$) and healthy individuals with positive tuberculin skin tests (TST$^+$ individuals; $n = 8$) or negative tuberculin skin tests (TST$^-$ individuals; $n = 2$). The protocol and consent forms describing the involvement of human subjects were approved by the New York University School of Medicine Institutional Review Board for the Bellevue Hospital. Whole PBMC were stimulated with each peptide for 10 to 14 days in the presence of interleukin-2 and interleukin-7 (4, 12, 21). Responding CTL were amplified by restimulation rounds with HLA-A*0201-expressing JY lymphoblastoid cells pulsed with peptides and allogeneic PBMC as feeder cells. Overall, there was no correlation between relative affinities of peptides for HLA-A*0201 and their abilities to amplify specific CTL responses (Table 1). In contrast, the stability of peptide-ligand complexes on the cell surface appears to be important because complexes with half-lives of less than 6 h were not recognized by CTL. CTL specific for the peptide comprising amino acids 160 to 168 of SodA ($SodA_{160-168}$) were found in three of eight TB patients and in two of eight TST$^+$ individuals, while $AlaDH_{160-169}$- and $GlnS_{308-316}$-specific CTL were absent in TB patients but present in two of eight and three of four TST$^+$ individuals, respectively. No CTL responses specific for these epitopes were detected in TST$^-$ individuals.

CTL assays demonstrated that the $SodA_{160-168}$, $AlaDH_{160-169}$, and $GlnS_{308-316}$ peptides are novel CTL epitopes as peptide-pulsed T2 cells were specifically and efficiently lysed by effectors present in stimulated PBMC from TB patients and TST$^+$ individuals (Fig. 1A to C). The efficiency of cytolysis was clearly increased after repeated stimulations with the peptides, thus suggesting a continuous enrichment with specific effectors in these cultures. The MHC class I restriction of generated CTL was confirmed with a panel of derivatives of the MHC class I-negative 721.221 cells expressing the HLA-G, HLA-B*2705, or HLA-A*0201 allele. As shown, only cells which expressed the HLA-A*0201 allele and were capable of presenting $SodA_{160-168}$, $AlaDH_{160-169}$, and $GlnS_{308-316}$ peptides were lysed

* Corresponding author. Mailing address: Laboratory of Molecular Immunology, Public Health Research Institute, 225 Warren Street, Newark, NJ 07103. Phone: (973) 854-3350. Fax: (973) 854-3101. E-mail: yuri@phri.org.

TABLE 1. CTL epitopes identified within HLA-A*0201-binding peptides derived from mycobacterial SodA, AlaDH, and GlnS proteins

Protein	Peptide[a]	Sequence	RA[b]	DC_{50}[c]	CTL activity[d]
SodA	160–168	DMWEHAFYL	15.6	6	Yes
	160–169	DMWEHAFYLQ	20.0	6	Yes
AlaDH	160–169	VLMGGVPGVE	1.8	>8	Yes
	344–353	GLSTHEGALL	11.0	4	No
	352–361	LLSERVATDL	20.0	4	No
GlnS	308–316	GLLHHAPSL	11.6	>8	Yes
	316–324	LLAFTNPTV	4.0	>8	No
	422–430	QLSDVIDRL	5.6	6	No

[a] The sequence numbers of the first and last amino acids are shown. CTL epitope predictions were made using SodA, AlaDH, and GlnS proteins (P17670, P30234, and Q10377 or rv3846, rv2780, and rv2220, respectively). The peptides with the highest scores in the EpiMatrix program (5) were then analyzed by the SYFPEITHI and AMPHI programs (14, 18).

[b] RA (relative affinity) is the ratio of the concentration of each peptide to the concentration (5 μM) of the reference peptide TAX_{11-19}, LLFGYPVYV, that induced 20% HLA-A*0201 expression on T2 cells in stabilization assays (24).

[c] DC_{50} (dissociation complex) is the time required for the loss of 50% of peptide-ligand HLA-A2 complexes stabilized at the T2 cell surface after overnight culture with 100 μM (each) peptides and 100 ng of β2-microglobulin/ml.

[d] The HLA-A*0201-positive TB patients and TST^+ healthy individuals were identified by immunostaining of PBMC with HLA-A2-specific MAbs MA2.1, PA2.1, and CR11-351 and flow cytometry analysis. The HLA-A2 subtyping was confirmed by the response of donor $CD8^+$ CTL to the influenza A virus matrix peptide FMP $M1_{58-66}$. PBMC were restimulated with peptides for 3 to 4 weeks and then tested for CTL activity against peptide-pulsed T2 target cells by using lactate dehydrogenase release ELISA (Roche Molecular Biochemicals, Mannheim, Germany). Specific lysis of greater than twofold of that obtained with an irrelevant TAX_{11-19} peptide was considered to indicate positivity for CTL activity. Stimulated PBMC from two HLA-A*0201-positive, TST^- donors had no peptide-specific CTL activity (data not shown).

(Fig. 1D to F). Moreover, the peptide-specific cytolysis of targets was inhibited by anti-MHC class I and anti-CD8 MAb but not by MAb to CD4, thus corroborating the MHC class I restriction and CD8 dependency of these effectors (Fig. 1G to I and data not shown).

To control mycobacterial growth, MHC class I-restricted $CD8^+$ CTL are expected to recognize and respond to their target epitopes presented by *M. tuberculosis*-infected cells during the natural course of infection. We next examined the ability of CTL specific for $SodA_{160-168}$, $AlaDH_{160-169}$, or $GlnS_{308-316}$ to recognize the endogenously generated epitopes and to exert effector functions against infected targets. Therefore, S1, A1, and G1 effectors were assayed for cytolysis of the HLA-A*0201-positive monocytic cell line THP-1 acutely infected with *M. tuberculosis* (4). All three peptide-specific effectors were able to recognize and lyse *M. tuberculosis*-infected THP-1 cells (Fig. 1K to M).

The importance of Th1 cytokines IFN-γ and tumor necrosis factor alpha (TNF-α) in immune response to *M. tuberculosis* infection is known (19). Accumulated data indicate that production of these cytokines is an essential effector function of $CD8^+$ T cells responding to the pathogen in humans and mice (10). We therefore investigated whether peptide-specific CTL can release IFN-γ and TNF-α upon recognition of their targets. First, using enzyme-linked immunosorbent assays (ELISA), we found that peptide-stimulated, CD8-enriched S1, A1, and G1 effectors released IFN-γ and TNF-α in the range of 0.8 to 1.5 ng/ml and 4.0 to 4.5 ng/ml, respectively (data not

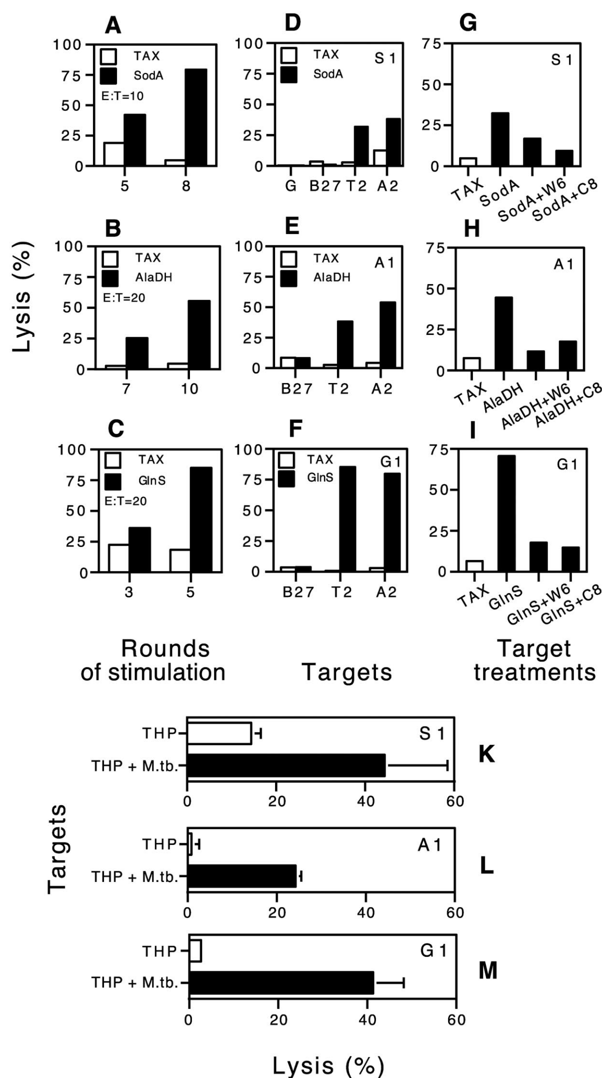

FIG. 1. HLA-A*0201-restricted and CD8-dependent CTL responses to synthetic and endogenously processed $SodA_{160-168}$, $AlaDH_{160-169}$, and $GlnS_{308-316}$ target peptides. (A to C) Enrichment with specific CTL in PBMC cultures from a TB patient and two TST^+ individuals (Table 1) after repeated stimulation with target peptides. CTL activity against T2 cells pulsed with each target or irrelevant TAX_{11-19} peptides at the indicated effector-to-target (E:T) ratios was measured using lactate dehydrogenase release ELISA. (D to I) The effector S1 line and A1 and G1 clones were tested against respective peptide-pulsed 721.221 transfectants expressing HLA-G (G), HLA-B*2705 (B27), or HLA-A*0201 (A2) alleles and HLA-A*0201-positive T2 cells or against target T2 cells in the presence of MAb to MHC class I (W6) and CD8 (C8). (K to M) Cytolysis of THP-1 cells acutely infected with *M. tuberculosis* H37Rv (THP + M.tb.) at 4 CFU per cell (40 to 50% infectivity by acid-fast staining) by S1, A1, and G1 effectors at an effector-to-target ratio of 20. Values are the means ± standard deviations of results from triplicate determinations. The composite is representative of results from at least three experiments.

shown). Next, the frequencies of cytokine producers among effectors were determined by intracellular staining. Induction of IFN-γ release was significantly more robust in AlaDH-specific CTL, which showed a high frequency (40%) of producers, than in SodA- and GlnS-specific CTL, which showed lower frequencies (6 to 7%) (Fig. 2). TNF-α producers were detected

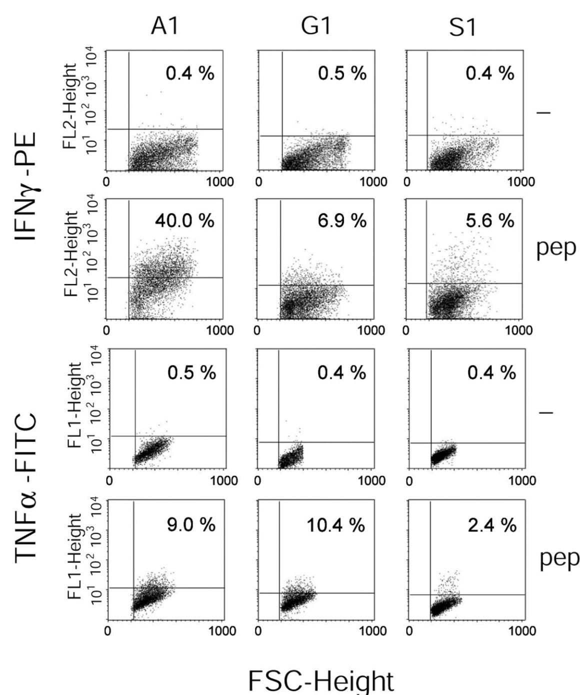

FIG. 2. Production of IFN-γ and TNF-α by SodA-, AlaDH-, and GlnS-specific CTL. S1, A1, and G1 effectors were stimulated with target peptide-pulsed (pep) or nonpulsed (−) T2 cells in the presence of brefeldin A and then tested for the frequency (in percent) of cytokine producers by intracellular staining with MAb to IFN-γ and TNF-α and flow cytometry analysis. The CTL were 100% $CD3^+$ and $CD8^+$. The histogram profiles were obtained after gating of viable lymphocytes. The total cytokine-producing potential assessed after stimulation with phorbol myristate acetate and ionomycin was 43 to 51%; that is, 43 to 51% of these effectors were IFN-γ and TNF-α producers (data not shown). The data are representative of results from three experiments. FITC, fluorescein isothiocyanate; PE, phycoerythrin; FL, fluorescence channel; FSC, forward scatter.

among all effectors, with frequencies of about 2 to 10%. Differences in frequencies of cytokine producers may reflect the presence of effectors that have similar lytic capabilities but that bear T-cell receptors with differential avidities for the peptide-ligand complexes and/or the presence of functionally distinct CTL not secreting IFN-γ and TNF-α (13, 27).

We have identified three novel immunodominant CTL epitopes, $SodA_{160\text{-}168}$, $AlaDH_{160\text{-}169}$, and $GlnS_{308\text{-}316}$, which are endogenously processed and presented by *M. tuberculosis*-infected cells. CTL precursors specific for these epitopes and possessing effector functions such as cytolysis and IFN-γ and TNF-α release can be isolated from peripheral blood of TB patients and/or TST^+ individuals. The proteins expressing these CTL epitopes are enzymes with defined roles in the metabolic processes in mycobacteria. In addition, they may be involved in host-pathogen interactions. Thus, SodA detoxifies superoxide anion and is capable of neutralizing oxygen metabolites released from immune cells. This is likely to have a broad effect on infection pathogenesis. Indeed, SodA has been shown to inhibit innate immune responses in a mouse model (6). AlaDH is involved in nitrogen metabolism and has been implicated in the adaptation of mycobacteria to an anaerobic dormant state, a condition that may be related to the state of latent infection (9). Finally, GlnS is similarly involved in another pathway of nitrogen metabolism and is essential for intracellular growth of bacilli in macrophages (25). Therefore, these enzymes appear to be crucial for the survival of mycobacteria and may be the targets of strong cell-mediated host immune responses, as revealed in our studies. Further studies are under way to determine whether the response to these CTL epitopes in TB patients and TST^+ individuals is a correlate of immunity to TB.

This work was supported by National Institutes of Health grant HL 59835 (Y.B.) and GCRC grant MO1 00096 (W.N.R.).

REFERENCES

1. **Andersen, A. B., P. Andersen, and L. Ljungqvist.** 1992. Structure and function of a 40,000-molecular-weight protein antigen of *Mycobacterium tuberculosis.* Infect. Immun. **60:**2317–2323.
2. **Andersen, P., D. Askgaard, A. Gottschau, J. Bennedsen, S. Nagai, and I. Heron.** 1992. Identification of immunodominant antigens during infection with *Mycobacterium tuberculosis.* Scand. J. Immunol. **36:**823–831.
3. **Caccamo, N., S. Milano, C. Di Sano, D. Cigna, J. Ivanyi, A. M. Krensky, F. Dieli, and A. Salerno.** 2002. Identification of epitopes of *Mycobacterium tuberculosis* 16-kDa protein recognized by human leukocyte antigen-A*0201 $CD8^+$ T lymphocytes. J. Infect. Dis. **186:**991–998.
4. **Cho, S., V. Mehra, S. Thoma-Uszynski, S. Stenger, N. Serbina, R. J. Mazzaccaro, J. L. Flynn, P. F. Barnes, S. Southwood, E. Celis, B. R. Bloom, R. L. Modlin, and A. Sette.** 2000. Antimicrobial activity of MHC class I-restricted $CD8^+$ T cells in human tuberculosis. Proc. Natl. Acad. Sci. USA **97:**12210–12215.
5. **De Groot, A. S., B. M. Jesdale, E. Szu, J. R. Schafer, R. M. Chicz, and G. Deocampo.** 1997. An interactive Web site providing major histocompatibility ligand predictions: application to HIV research. AIDS Res. Hum. Retrovir. **13:**529–531.
6. **Edwards, K. M., M. H. Cynamon, R. K. R. Voladri, C. C. Hager, M. S. DeStefano, K. T. Tham, D. L. Lakey, M. R. Bochan, and D. S. Kernodle.** 2001. Iron-cofactored superoxide dismutase inhibits host responses to *Mycobacterium tuberculosis.* Am. J. Respir. Crit. Care Med. **164:**2213–2219.
7. **Flynn, J. L., and J. Chan.** 2001. Immunology of tuberculosis. Annu. Rev. Immunol. **19:**93–129.
8. **Geluk, A., K. E. van Meijgaarden, K. L. Franken, J. W. Drijfhout, S. D'Souza, A. Necker, K. Huygen, and T. H. Ottenhoff.** 2000. Identification of major epitopes of *Mycobacterium tuberculosis* AG85B that are recognized by HLA-A*0201-restricted $CD8^+$ T cells in HLA-transgenic mice and humans. J. Immunol. **165:**6463–6471.
9. **Hutter, B., and T. Dick.** 1998. Increased alanine dehydrogenase activity during dormancy in *Mycobacterium smegmatis.* FEMS Microbiol. Lett. **167:**7–11.
10. **Kerksiek, K. M., and E. G. Pamer.** 1999. T cell responses to bacterial infection. Curr. Opin. Immunol. **11:**400–405.
11. **Klein, M. R., S. M. Smith, A. S. Hammond, G. S. Ogg, A. S. King, J. Vekemans, A. Jaye, P. T. Lukey, and K. P. McAdam.** 2001. HLA-B*35-restricted CD8 T cell epitopes in the antigen 85 complex of *Mycobacterium tuberculosis.* J. Infect. Dis. **183:**928–934.
12. **Lalvani, A., R. Brookes, R. J. Wilkinson, A. S. Malin, A. A. Pathan, P. Andersen, H. Dockrell, G. Pasvol, and A. V. Hill.** 1998. Human cytolytic and interferon γ-secreting $CD8^+$ T lymphocytes specific for *Mycobacterium tuberculosis.* Proc. Natl. Acad. Sci. USA **95:**270–275.
13. **Lawson, T. M., S. Man, E. C. Y. Wang, S. Williams, N. Amos, G. M. Gillespie, P. A. Moss, and L. K. Borysiewicz.** 2001. Functional differences between influenza A-specific cytotoxic T lymphocyte clones expressing dominant and subdominant TCR. Int. Immunol. **13:**1383–1390.
14. **Margalit, H., J. L. Spouge, J. L. Cornette, K. B. Cease, C. Delisi, and J. A. Berzovsky.** 1987. Prediction of immunodominant helper T cell antigenic sites from the primary sequence. J. Immunol. **138:**2213–2229.
15. **Mohagheghpour, N., D. Gammon, L. M. Kawamura, A. van Vollenhoven, C. J. Benike, and E. G. Engleman.** 1998. CTL response to *Mycobacterium tuberculosis*: identification of an immunogenic epitope in the 19-kDa lipoprotein. J. Immunol. **161:**2400–2406.
16. **Nijman, H. W., J. G. Houbiers, M. P. Vierboom, S. H. Van der Burg, J. W. Drijfhout, J. D'Amaro, P. Kenemans, C. J. Melief, and W. M. Kast.** 1993. Identification of peptide sequences that potentially trigger HLA-A2.1-restricted cytotoxic T lymphocytes. Eur. J. Immunol. **6:**1215–1219.
17. **Pathan, A. A., K. A. Wilkinson, R. J. Wilkinson, M. Latif, H. McShane, G. Pasvol, A. V. Hill, and A. Lalvani.** 2000. High frequencies of circulating IFN-γ-secreting CD8 cytotoxic T cells specific for a novel MHC class I-restricted *Mycobacterium tuberculosis* epitope in *M. tuberculosis*-infected subjects without disease. Eur. J. Immunol. **30:**2713–2721.
18. **Rammensee, H., J. Bachmann, N. P. Emmerich, O. A. Bachor, and S.**

Stevanovic. 1999. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics **50:**213–219.
19. **Raupach, B., and S. H. Kaufmann.** 2001. Immune responses to intracellular bacteria. Curr. Opin. Immunol. **13:**417–428.
20. **Seder, R. A., and A. V. S. Hill.** 2000. Vaccines against intracellular infections requiring cellular immunity. Nature **406:**793–798.
21. **Smith, S. M., R. Brookes, M. R. Klein, A. S. Malin, P. T. Lukey, A. S. King, G. S. Ogg, A. V. Hill, and H. M. Dockrell.** 2000. Human $CD8^+$ CTL specific for the mycobacterial major secreted antigen 85A. J. Immunol. **165:**7088–7095.
22. **Sonnenberg, M. G., and J. T. Belisle.** 1997. Definition of *Mycobacterium tuberculosis* culture filtrate proteins by two-dimensional polyacrylamide gel electrophoresis, N-terminal amino acid sequencing, and electrospray mass spectrometry. Infect. Immun. **65:**4515–4524.
23. **Stenger, S., D. A. Hanson, R. Teitelbaum, P. Dewan, K. R. Niazi, C. J. Froelich, T. Ganz, S. Thoma-Uszynski, A. Melian, C. Bogdan, S. A. Porcelli, B. R. Bloom, A. M. Krensky, and R. L. Modlin.** 1998. An antimicrobial activity of cytolytic T cells mediated by granulysin. Science **282:**121–125.
24. **Tourdot, S., A. Scardino, W. Sllaloustrou, D. A. Gross, S. Pascolo, P. Cordopatis, F. A. Lemonnier, and K. Kosmatopoulos.** 2000. A general strategy to enhance immunogenicity of low-affinity HLA-A2.1-associated peptides: implication in the identification of cryptic tumor epitopes. Eur. J. Immunol. **30:**3411–3421.
25. **Tullius, M. V., G. Harth, and M. A. Horwitz.** 2003. Glutamine synthetase GlnA1 is essential for growth of *Mycobacterium tuberculosis* in human THP-1 macrophages and guinea pigs. Infect. Immun. **71:**3927–3936.
26. **Tullius, M. V., G. Harth, and M. A. Horwitz.** 2001. High extracellular levels of *Mycobacterium tuberculosis* glutamine synthetase and superoxide dismutase in actively growing cultures are due to high expression and extracellular stability rather than to a protein-specific export mechanism. Infect. Immun. **69:**6348–6363.
27. **Valitutti, S., S. Muller, M. Dessing, and A. Lanzavecchia.** 1996. Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. J. Exp. Med. **183:**1917–1921.

Editor: S. H. E. Kaufmann