Serum Angiotensin-1 Converting Enzyme Activity Processes a Human Immunodeficiency Virus 1 gp160 Peptide for Presentation by Major Histocompatibility Complex Class I Molecules

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Summary

T cell stimulation by the human immunodeficiency virus 1 gp160-derived peptide p18 presented by H-2D^d class I major histocompatibility complex molecules in a cell-free system was found to require proteolytic cleavage. This extracellular processing was mediated by peptidases present in fetal calf serum. In vitro processing of p18 resulted in a distinct reverse phase high performance liquid chromatography profile, from which a biologically active product was isolated and sequenced. This peptide processing can be specifically blocked by the angiotensin-1 converting enzyme (ACE) inhibitor captopril, and can occur by exposing p18 to purified ACE. The ability of naturally occurring extracellular proteases to convert inactive peptides to T cell antigens has important implications for understanding cytotoxic T lymphocyte responses in vivo, and for rational peptide vaccine design.

T lymphocytes that express CD4 accessory molecules generally recognize an antigenic fragment of an exogenous protein bound to a class II MHC molecule. In contrast, CD8$^+$ T cells generally recognize an antigenic fragment of an endogenous protein bound to a class I molecule (1, 2). Exceptions to this model, where exogenous proteins are processed and presented on class I MHC molecules (3–5), may play a role in aberrant immune responses and may be useful for design of noninfectious CTL-inducing vaccines. Specific extracellular peptidases can favor generation or destruction of optimally sized (6–10) class I-binding peptides from larger protein fragments. This processing can explain the efficacy of many peptides that exceed the optimal length in in vitro experiments, and is one possible mechanism by which exogenous proteins or peptides can sensitize cells to class I-restricted lysis.

Materials and Methods

Peptides and Peptide Sequencing. The HIV gp160 envelope glycoprotein-derived peptides, p18 (RIQRGPGRAFVTIGK) (11) and p18-I-10 (RGPGRAFVTI) (T. Takeshita, manuscript in preparation), were synthesized by t-BOC chemistry and purified by reverse phase HPLC. Recovered HPLC fractions were sequenced by automated Edman degradation on a model 470A sequenator and fractions were identified by amino acid analysis on a model 120A PTH analyzer (both from Applied Biosystems, Inc., Foster City, CA).

Soluble H-2D^d Class I Protein. Soluble H-2D^d protein was affinity purified from supernatants of L cells transfected with an H-2D^d genomic construct consisting of DNA encoding the H-2D^d $\alpha_1\alpha_2\alpha_3$ domains and the 27 COOH-terminal amino acid residues of the soluble Q10^b molecule (12).

Exopeptidases and Inhibitors. Carboxypeptidase N (EC 3.4.17.3) (Calbiochem Corp., La Jolla, CA), ACE[1] angiotensin-1 converting enzyme (EC 3.4.15.1), captopril (both from Sigma Chemical Co., St. Louis, MO), and potato carboxypeptidase inhibitor (Calbiochem Corp.) were dissolved in PBS. Plummer's inhibitor was dissolved in acidified deionized water. E-64 (both from Calbiochem Corp.) was dissolved in 33% DMSO (final concentration was always <1.7%).

HPLC Fractionation of p18. 40 μl of 2.5 mM p18 was added to 160 μl 1% OVA or 1% BSA for 15 h at 37°C. 100 μl of each sample was spun through a Centricon 3 filter (Amicon, Beverly, MA) into 100 μl of 1% BSA. The samples were injected into a 4.6 mm × 250 mm C18 reverse phase column (Vydac, Hesperia,

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CA), and eluted with a gradient of 15-30% acetonitrile over 30 min. The flow rate was 1 ml/min and the absorbance was monitored at 220 nm. Forty 1-ml fractions were collected, dried down in a Spin-Vac, and resuspended in 200 μl deionized water. The pooled and unpooled fractions were diluted 1:8 in 0.1% BSA in PBS, filter-sterilized and evaluated for functional activity.

Stimulation of the T Cell Hybridoma with Purified Class I. Soluble H-2D^d protein was coated onto Immulon 4 plates (Dynatech Labs., Inc., Chantilly, VA) at 0.1-0.25 μg/well in 50-μl PBS for 2-2.5 h at 37°C. The plates were washed twice with 200-μl PBS and blocked for 30-60 min with the incubation medium to be added during the peptide pulsing period.

Peptide and human β$_2$-microglobulin (β$_2$-m) (Calbiochem Corp.) (0.2 μg/well) were added to the incubation media, 0.5% BSA (Sigma Chemical Co. fraction V), OVA (Sigma Chemical Co. grade V), or FCS (Hyclone Laboratories Inc., Logan, UT), to give a final volume of 200 μl/well, and the plates were incubated at 37°C and 7.5% CO_2 for 22-26 h. The plates were then washed twice with PBS and 2 × 10^4 B4.2.3 T-hybridoma cells (anti-p18 plus H-2D^d) (12) were added per well in DMEM supplemented with 10% FCS, 2-mM glutamine, nonessential amino acids, 50 μg/ml gentamicin, and 5 × 10^{-5} M 2-ME (complete medium). The plates were incubated from 16-20 h at 37°C and 7.5% CO_2. The cells were then pulsed with 1-μCi [^{3}H]thymidine (ICN Biomedicals, Inc., Costa Mesa, CA), and collected 4-8 h later for counting the amount of incorporated label to evaluate growth inhibition (13).

Response of T Cell Hybridoma to Cell-surface Class I. H-2D^d positive L cells (2 × 10^4/well) with peptide, 3T3 neomycin resistance gene transfectant cells, or 3T3 gp160 gene transfectant cells were added to each well with 10^4 B4.2.3 T-hybridoma cells. After an overnight incubation, 50 μl/well of supernatant was harvested and freeze-thawed. These supernatants were added to 4 × 10^3 CTLL-2 cells in RPMI complete medium to give a final volume of 200 μl/well. After an 18-h incubation, 1-μCi [^{3}H]thymidine was added to each well. 4 h later, the CTLL-2 cells were collected and counted for incorporated thymidine.

Results and Discussion

The HIV-1 (IIIB) gp160 envelope glycoprotein-derived peptide, p18, is 15 amino acids in length (residues 315-329). It is the immunodominant CTL determinant of gp160 in H-2D^d mice (11, 14), and is also able to sensitize syngeneic cells for lysis by CTL from HIV-1-infected humans (15). Previous studies of the ability of this peptide to form a stimulatory complex with soluble purified H-2D^d molecules in vitro, indicated that two activities of FCS were required for recognition of p18 by a specific T cell hybridoma. One activity was that of β$_2$-m (12, 16-19), and the other activity could be provided by OVA. Most batches of BSA were unable to replace this β$_2$-m-independent effect of FCS. The recent evidence concerning the importance of length in the activity of peptides presented by MHC class I molecules, and the identification of a truncation of p18, p18-I-10 (residues 318-327), with 10^1-10^2-fold greater potency of T cell stimulation (T. Takeshita, manuscript in preparation) prompted us to consider the possibility that OVA and FCS were processing p18 to an active, shorter peptide.

Thus, we compared the two peptides of different lengths in our cell-free system for their functional binding to class I MHC molecules in the presence of BSA, OVA, or FCS (Fig. 1). This binding was evaluated through activation of the B4.2.3 p18-specific T cell hybridoma, measured by growth inhibition (13). With p18, FCS or OVA was required for significant activation of B4.2.3. In contrast, this activation was decreased by FCS or OVA when p18-I-10 was used. The concentration of p18-I-10 which gave half-maximal stimulation was 10^{-11} M when added in BSA. This concentration was 10^1-10^2-fold less than the half-maximal concentration of p18-I-10 used in FCS, and 10^3-fold lower than the half-maximal concentration of p18-I-10 when used in OVA.

One possible explanation of these results is that proteolytic enzymes in OVA and FCS degrade the p18 15mer to a smaller active form, and also reduce the active 18-I-10 10mer to an inactive form. To evaluate this hypothesis, we incubated p18 with either OVA or BSA overnight, size-fractionated the small MW peptides away from the OVA or BSA, and analyzed them by reverse-phase HPLC (Fig. 2). A decrease in the amount, and a slight increase of the retention time of
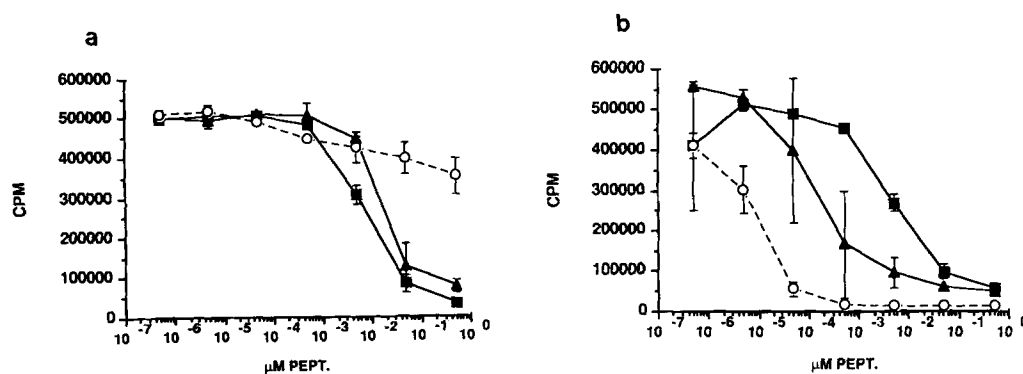

Figure 1. (a) B4.2.3 response to p18 is dependent on OVA or FCS. The functional response assayed by growth inhibition was measured as described in Materials and Methods. (--O--) p18 in 0.5% BSA; (--■--) p18 in 0.5% OVA; (--▲--) p18 in 0.5% FCS. (b) B4.2.3 growth inhibition response to p18-I-10 is decreased by OVA or FCS. (--O--) 18-I-10 in 0.5% BSA; (--■--) 18-I-10 in 0.5% OVA; (--▲--) 18-I-10 in 0.5% FCS. No peptide in BSA, 475,500 cpm ± 5710 sem. No peptide in OVA, 512,800 cpm ± 34,400 sem. No peptide in FCS, 509,900 cpm ± 3,530 sem. Results are expressed as cpm ± sem. This experiment was done in duplicate, and similar results were obtained in three experiments.
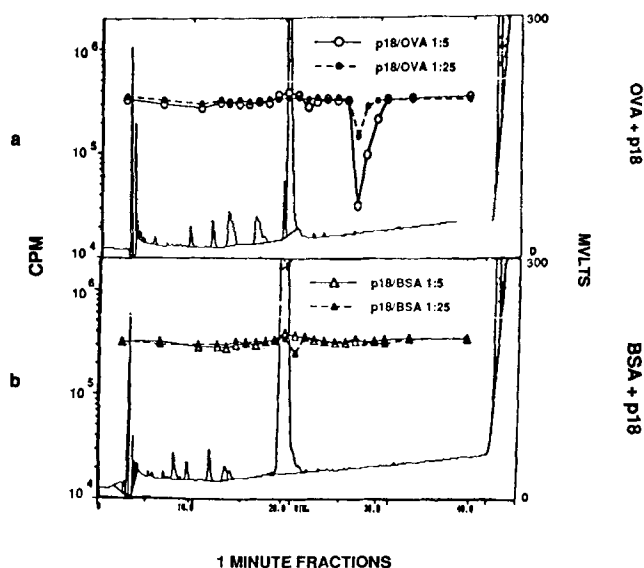
1 MINUTE FRACTIONS

Figure 2. HPLC fractions of treated p18 were collected and evaluated for growth inhibition of B4.2.3 as described in Materials and Methods. (*a*) Reverse phase HPLC fractions of OVA-treated p18 and their ability to functionally bind H-2D^d. (━━━━) 220-nm absorbance; (–O–) response of B4.2.3 to 1:5 dilution of fractions in 0.5% BSA added to H-2D^d coated plates; (–●–) 1:25 dilution of fractions in 0.5% BSA added to H-2D^d coated plates. B4.2.3 incorporated 345,000 cpm with H-2D^d incubated with 0.5% BSA. B4.2.3 incorporated 11,900 cpm with H-2D^d incubated with 0.05 μM 18-I-10 in 0.5% BSA. (*b*) Reverse phase HPLC fractions of BSA-treated p18 and their ability to functionally bind H-2D^d. (━━━━) 220-nm absorbance; (–△–) response of B4.2.3 to 1:5 dilution of fractions in 0.5% BSA added to H-2D^d coated plates; (–▲–) 1:25 dilution of fractions in 0.5% BSA added to H-2D^d coated plates. B4.2.3 incorporated 384,000 cpm with H-2D^d incubated with 0.5% BSA. B4.2.3 incorporated 9,649 cpm with H-2D^d incubated with 0.05 μM 18-I-10 in 0.5% BSA. Similar results were obtained in two experiments.

the major peak of p18 in PBS (data not shown) was seen in the OVA-treated peptide, but not in the BSA-treated peptide. The HPLC profile of the OVA-treated p18 also differed from the BSA-treated p18 in amount and retention time of several minor peaks. To determine in which fractions of the OVA-treated p18 the T cell–stimulatory activity eluted, the fractions were assayed for presentation by plate-bound H-2D^d. The active growth-inhibiting material was in fractions 26 and 27, eluting later than the p18 major peak. These fractions had very little 220-nm absorbance. The BSA-treated p18 fractions were unable to inhibit the growth of the T cell hybridoma. This observation suggested that a very small fraction of the processed p18 was a highly active peptide, as has been noted for the SV12 peptide and its synthetic contaminants (8). However, in contrast to the latter case, this active peptide is not a contaminant of the original p18 preparation. The active fractions were pooled and the sequence of the peptide was determined to be XIQRGPGRAFVTI, which is identical to p18 lacking two COOH-terminal residues. A clear peptide sequence was difficult to obtain from the active fraction generated by FCS treatment of p18, probably because of a more complex proteolytic system and contaminating serum peptides. The activity in the OVA appeared to be that

of a carboxypeptidase, removing the two COOH-terminal residues from p18.

To identify the carboxypeptidase that processes p18 in FCS, we titrated four carboxypeptidase inhibitors into p18 FCS mixtures, adding them to plate-bound H-2D^d. The inhibitors used were potato carboxypeptidase inhibitor (20), which blocks tissue carboxypeptidases A and B, Plummer's inhibitor (21), which blocks carboxypeptidase N (serum carboxypeptidase B), captopril (22), which blocks ACE or peptidyl dipeptidase A, and E-64 (23), which blocks cathepsin B (peptidyl dipeptidase B). In the presence of FCS, nanomolar concentrations of captopril blocked p18-dependent stimulation of B4.2.3 (Fig. 3 *a*). The blocking of FCS processing of p18 occurred at captopril concentrations 10^4–10^5-fold lower than that of any of the other carboxypeptidase inhibitors. This result suggested ACE (24, 25), a key regulator of vasoactive peptides, as a major serum processor of p18. Thus, we attempted to process p18 in the absence of serum or OVA using rabbit lung ACE (Fig. 3 *b*). The purified ACE was able to process p18 without serum, whereas human carboxypeptidase N was unable to do so. ACE was not required for T cell hybridoma stimulation by p18-I-10, and had some inhibitory effect at high concentrations using both p18 and p18-I-10.

We then evaluated whether the role of ACE in processing p18 for presentation by purified class I molecules would also apply to cell-surface class I molecules. This experiment was done using H-2D^d–transfected L cells as the antigen presenting cells. Peptides p18 and p18-I-10 were titrated in the presence of 10^{-5} M captopril or Plummer's inhibitor in the presence of medium containing FCS (Fig. 4 *a*). The p18 concentration required for half-maximal lymphokine production by the hybridoma was increased by 10^3–10^4-fold in the presence of captopril. Captopril had no significant effect on p18-I-10, excluding a direct cellular effect of captopril, and Plummer's inhibitor and E-64 (data not shown) had no effect on p18. In contrast, stimulation of the B4.2.3 hybridoma is not affected by captopril or Plummer's inhibitor when a transfectant cell (11) that expresses the gp160 envelope protein and H-2D^d is used as the antigen source (Fig. 4 *b*). The data in Fig. 4 *a* suggest that the ACE extracellular processing demonstrated in the cell-free system is applicable to the cell-surface system. The data in Fig. 4 *b* suggest that the intracellular processing of the whole antigen is not dependent on an ACE-like activity, or occurs in a cellular compartment inaccessible to captopril.

We have demonstrated that ACE activity is required for the processing of a long peptide (15 residues), and that serum can inactivate a short peptide (10 residues). ACE can remove COOH-terminal dipeptides from many substrates at varying rates. However, peptides with a penultimate proline are not cleaved (25). The serum processing of a peptide to the correct COOH-terminus for MHC binding would depend on the accumulation of a particular kinetic intermediate in proteolysis. The observation that the active peptide produced by OVA's exopeptidases is a 13mer with the same COOH terminus as the active synthetic peptide 18-I-10, suggests the
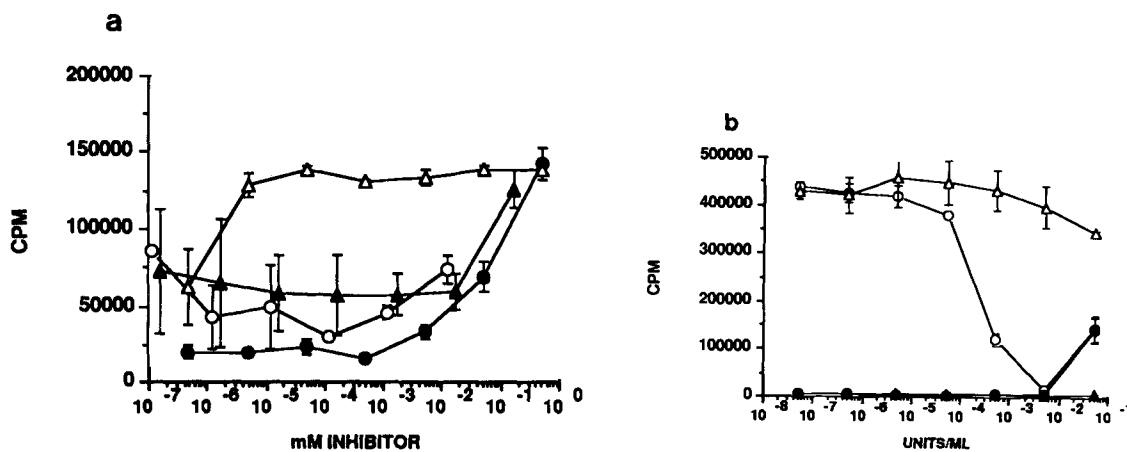
Figure 3. Effect of carboxypeptidase inhibitors and carboxypeptidase on p18 processing. (a) Effect of carboxypeptidase inhibitors on p18 functional binding to H-2D^d in FCS. Growth inhibition in the presence of (—O—) potato carboxypeptidase inhibitor; (—●—) Plummer's inhibitor; (—△—) captopril; (—▲—) E-64 was measured as described in Materials and Methods. B4.2.3 thymidine incorporation without inhibitors. No peptide in FCS 145,000 cpm ± 11,200 sem; 1 μM p18 in FCS 27,800 cpm ± 4,400 sem. No peptide in BSA 145,000 cpm ± 7,000 sem; 1 μM p18 in BSA 146,000 cpm ± 1,600 sem. The carboxypeptidase inhibitors were titrated as shown in 1 μM p18 and 0.5% FCS. The experiment was done in triplicate and results are shown ± sem. Similar results were obtained in three experiments. (b) ACE processes p18 into an active form in BSA. Growth inhibition was assessed as above. (—O—) p18 plus ACE; (—●—) 18-I-10 plus ACE; (—△—) p18 plus carboxypeptidase N; (—▲—) 18-I-10 plus carboxypeptidase N. B4.2.3 thymidine incorporation without carboxypeptidases. No peptide in FCS 429,600 cpm ± 21,000 sem. No peptide in BSA 411,800 cpm ± 29,200 sem; 1 μM p18 in FCS 112,600 ± 13,200 sem; 1 μM p18 in BSA 449,600 cpm ± 21,600 sem; 0.1 μM 18-I-10 in FCS 13,100 cpm ± 1,000 sem; 0.1 μM 18-I-10 in BSA 5,500 cpm ± 120 sem. Carboxypeptidase N and ACE were titrated in 0.5% BSA. The experiment was done in triplicate and results are shown ± sem. Similar results were obtained in two experiments.
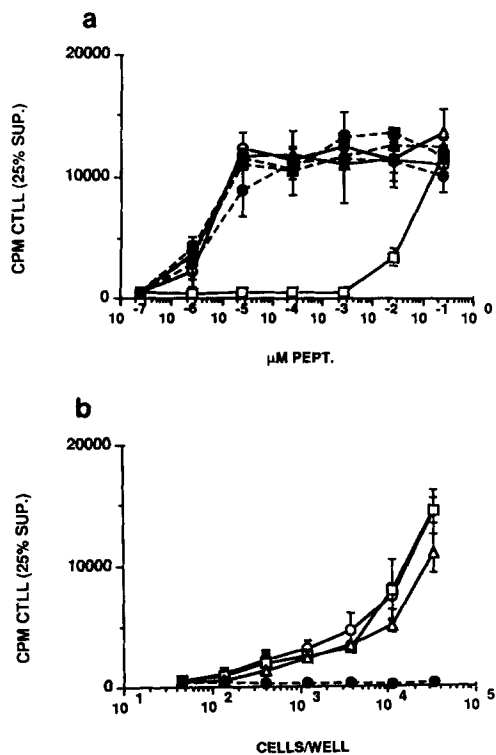

Figure 4. Effect of captopril on p18 and gp160 processing and presentation by cell-surface class I. (a) The B4.2.3 lymphokine response to p18 and H-2D^d positive L cells in FCS is significantly decreased by captopril. (—O—) p18; (—□—) p18 plus captopril; (—△—) p18 plus Plummer's inhibitor; (---●---) 18-I-10; (---■---) 18-I-10 plus captopril; (---▲---) 18-I-10 plus Plummer's inhibitor. CTLL-2 thymidine incorporation in the absence of peptide was <600 cpm. Captopril, Plummer's inhibitor, or no carbox-

key processing event contributed by OVA or FCS occurs at the COOH terminus. Other peptidases might be involved either before or after class I binding as suggested by Falk et al. (26). The ACE inhibitor captopril can increase or decrease the stimulation of a T cell hybridoma specific for a murine cytomegalovirus (MCMV) pp89 peptide depending on the COOH-terminal sequence flanking the core peptide (data not shown). The role of ACE and exopeptidases in general needs to be evaluated with different peptide fragments of gp120, and other class I-restricted antigens.

There are a number of reports in the literature regarding exogenous antigens being recognized by class I-restricted T cells, either through extracellular processing or specialized class I presenting cells (3, 5, 27, 28). ACE and other peptidases may trim fragments of soluble antigenic proteins in vivo. The availability of fragments of soluble antigenic proteins may depend on specialized cells that can release proteases or endocytose whole soluble antigen and release fragments (29, 30).

ypeptidase inhibitor was added to give a final concentration of 10^{-5} M and peptide (p18 or 18-I-10) was titrated. Results are shown as triplicates ± sem. Three experiments gave similar results. (b) The B4.2.3 lymphokine response to gp-160 transfected H-2D^d positive 3T3 cells is not decreased by captopril. (—O—) gp160 transfectant; (—□—) gp-160 transfectant plus captopril; (—△—) gp-160 transfectant plus Plummer's inhibitor; (---●---) neo transfectant. CTLL-2 thymidine incorporation in the absence of tranfected L cells was <500 cpm. Captopril, Plummer's inhibitor, or no carboxypeptidase inhibitor were added to give a final concentration of 10^{-5} M, and transfectant 3T3 cells were titrated as shown. Results are shown as triplicates ± sem. Three experiments gave similar results.

Extracellular processing of class I-restricted viral antigenic peptides may lead to generation of noninfected false targets competing for the CTL response against virally infected cells. This processing may also amplify a CTL response beneficial to the host. Serum enzymes can also lead to degradation of class I antigenic peptides (31). Pathogens could potentially follow different clinical courses based on their antigenic protein's ability to be processed or degraded extracellularly. PBLs from HIV-infected patients can recognize p18 presented by syngeneic cells (15). Since ACE inhibitors are currently being used for their hemodynamic benefits in the treatment of HIV cardiomyopathy (32), it will be useful to evaluate patients for the possible immunologic effects of this class of drugs.

A number of laboratories are investigating the use of synthetic peptides for immunization or other immunomodulatory effects. Understanding the specificity of peptide processing in human plasma and other body fluids is of vital importance in the direct design of these synthetic peptides and the possible use of peptidase inhibitors in conjunction with them. This understanding could lead to the development of highly effective vaccines.

References

1. Germain, R.N. 1986. Immunology. The ins and outs of antigen processing and presentation. *Nature (Lond.).* 322:687.
2. Braciale, T.J., L.A. Morrison, M.T. Sweetser, J. Sambrook, M.J. Gething, and V.L. Braciale. 1987. Antigen presentation pathways to class I and class II MHC-restricted T lymphocytes. *Immunol. Rev.* 98:95.
3. Staerz, U.D., H. Karasuyama, and A.M. Garner. 1987. Cytotoxic T lymphocytes against a soluble protein. *Nature (Lond.).* 329:449.
4. Carbone, R.F., N.A. Hosken, M.W. Moore, and M.J. Bevan. 1989. Class I MHC-restricted cytotoxic responses to soluble protein antigen. *Cold Spring Harbor Symp. Quant. Biol.* 54:551.
5. Rock, K.L., S. Gamble, and L. Rothstein. 1990. Presentation of exogenous antigen with class I major histocompatibility complex molecules. *Science (Wash. DC).* 249:918.
6. Van Bleek, G.M., and S.G. Nathenson. 1990. Isolation of an endogenously processed immunodominant viral peptide from the class IH-2Kb molecule. *Nature (Lond.).* 348:213.
7. Rötzschke, O., K. Falk, K. Deres, H. Schild, M. Norda, J. Metzger, G. Jung, and H.G. Rammensee. 1990. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature (Lond.).* 348:252.
8. Schumacher, T.N., M.L. De Bruijn, L.N. Vernie, W.M. Kast, C.J. Melief, J.J. Neefjes, and H.L. Ploegh. 1991. Peptide selection by MHC class I molecules. *Nature (Lond.).* 350:703.
9. Madden, D.R., J.C. Gorga, J.L. Strominger, and D.C. Wiley. 1991. The structure of HLA-B27 reveals nonamer self-peptides bound in an extended conformation. *Nature (Lond.).* 353:321.
10. Jaredetzky, T.S., W.S. Lane, R.A. Robinson, D.R. Madden, and D.C. Wiley. 1991. Identification of self peptides bound to purified HLA-B27. *Nature (Lond.).* 353:326.
11. Takahashi, H., J. Cohen, A. Hosmalin, K.B. Cease, R. Houghten, J.L. Cornette, C. DeLisi, B. Moss, R.N. Germain, and J.A. Berzofsky. 1988. An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 85:3105.
12. Kozlowski, S., T. Takeshita, W.H. Boehncke, H. Takahashi, L.F. Boyd, R.N. Germain, J.A. Berzofsky, and D.H. Margulies. 1991. Excess β_2-microglobulin promoting functional peptide associated with purified soluble class I MHC molecules. *Nature (Lond.).* 349:74.
13. Ashwell, J.D., R.E. Cunningham, P.D. Noguchi, and D. Hernandez. 1987. Cell growth cycle block of T cell hybridomas upon activation with antigen. *J. Exp. Med.* 165:173.
14. Takahashi, H., R. Houghten, S.D. Putney, D.H. Margulies, B. Moss, R.N. Germain, and J.A. Berzofsky. 1989. Structural requirements for class I MHC molecule-mediated antigen presentation and cytotoxic T cell recognition of an immunodominant determinant of the human immunodeficiency virus envelope protein. *J. Exp. Med.* 170:2023.
15. Clerici, M., D.R. Lucey, R.A. Zajac, R.N. Boswell, H.M. Gebel, H. Takahashi, J.A. Berosfsky, and G.M. Shearer. 1991. Detection of cytotoxic T lymphocytes specific for synthetic peptides of gp160 in HIV-seropositive individuals. *J. Immunol.* 146:2214.
16. Rock, K.L., L.E. Rothstein, S.R. Gamble, and B. Benacerraf. 1990. Reassociation with β_2-microglobulin is necessary for Kb class I major histocompatibility complex binding of exogenous peptides. *Proc. Natl. Acad. Sci. USA.* 87:7517.
17. Vitiello, A., T.A. Potter, and L.A. Sherman. 1990. The role of β_2-microglobulin in peptide binding by class I molecules. *Science (Wash. DC).* 250:1423.

18. Rock, K.L., S. Gamble, L. Rothstein, and B. Benacerraf. 1991. Reassociation with β_2-microglobulin is necessary for Db class I major histocompatibility complex binding of an exogenous influenza peptide. *Proc. Natl. Acad. Sci. USA.* 88:301.

19. Kane, K.P., L.A. Sherman, and M.F. Mescher. 1991. Exogenous β_2-microglobulin is required for antigenic peptide binding to isolated class I major histocompatibility complex molecules. *Eur. J. Immunol.* 21:2289.

20. Hasse, G.M., and C.A. Ryan. 1981. Carboxypeptidase inhibitor from potatoes. *Methods Enzymol.* 80:778.

21. Plummer, T.H.J., and T.J. Ryan. 1981. A potent mercapto biproduct analogue inhibitor for human carboxypeptidase N. *Biochem. Biophys. Res. Commun.* 98:448.

22. Cushman, D.W., H.S. Cheung, E.F. Sabo, and M.A. Ondetti. 1977. Design of potent competitive inhibitors of angiotensin-converting enzyme. Carboxyalkanoyl and mercaptoalkanoyl amino acids. *Biochemistry.* 16:5484.

23. Barrett, A.J., A.A. Kembhavi, M.A. Brown, H. Kirschke, C.G. Knight, M. Tamai, and K. Hanada. 1982. L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H, and L. *Biochem. J.* 201:189.

24. Ng, K.K.F., and J.R. Vane. 1968. Fate of angiotensin I in the circulation. *Nature (Lond.).* 218:144.

25. McDonald, J.K., and A.J. Barrett. 1986. Peptidyl dipeptidase

A. *In* Mammalian Proteases: A Glossary and Bibliography. Exopeptidases. Vol 2. Academic Press Limited. London. 227–50.

26. Falk, K., O. Rötzschke, and H.G. Rammensee. 1990. Cellular peptide composition governed by major histocompatibility complex class I molecules. *Nature (Lond.).* 348:248.

27. Moore, M.W., F.R. Carbone, and M.J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell.* 54:777.

28. Carbone, F.R., and M.J. Bevan. 1990. Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. *J. Exp. Med.* 171:377.

29. Semple, J.W., J. Ellis, and T.L. Delovitch. 1989. Processing and presentation of insulin. II. Evidence for intracellular, plasma membrane-associated and extracellular degradation of human insulin by antigen-presenting B cells. *J. Immunol.* 142:4184.

30. Diment, S., K.J. Martin, and P.D. Stahl. 1989. Cleavage of parathyroid hormone in macrophage endosomes illustrates a novel pathway for intracellular processing of proteins. *J. Biol. Chem.* 264:13403.

31. Widmann, C., J.L. Maryanski, P. Romero, and G. Corradin, and. 1990. Differential stability of antigenic MHC class I-restricted synthetic peptides. *J. Immunol.* 147:3745.

32. Kaul, S., M.C. Fishbein, and R.J. Siegel. 1991. Cardiac manifestations of acquired immune deficiency syndrome: a 1991 update. *Am. Heart J.* 122:535.