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Immunology

Antigen conformation determines processing requirements for T-cell activation

(myoglobin/antigen presentation/T-cell clones/lysosomotrophic agents/proteolysis)

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ABSTRACT **We studied the difference in requirements for processing and presentation to a single T-cell clone of four different forms of the same epitope of sperm whale myoglobin—namely, on the native protein, on two conformationally altered forms of the protein, or as a 22-residue antigenic peptide fragment. The T-cell clone was *I-E*^{d}-restricted and specific for an epitope on the CNBr fragment 132–153 involving Lys-140. As inhibitors of macrophage processing of antigen, we used several agents that inhibit lysosomal function: the weak bases chloroquine and NH_4Cl, the cationic ionophore monensin, and the competitive protease inhibitor leupeptin. When these agents were used to inhibit processing of antigen by presenting cells and then washed out before T cells were added to culture, they inhibited the presentation of native antigen but not of fragment 132–153. To our surprise, the intact but denatured form, *S*-methylmyoglobin, behaved like the fragment not like the native protein. Apomyoglobin was intermediate in susceptibility to inhibition. Thus, native myoglobin requires a processing step that appears to involve lysosomal proteolysis, which is not required by fragment 132–153 or the denatured unfolded forms. For an antigen the size of myoglobin (M_r 17,800), it appears that unfolding of the native conformation, rather than further reduction in size, is the critical parameter determining the need for processing. Since a major difference between native myoglobin and the other forms is the greater accessibility in the latter of sites, such as hydrophobic residues, buried in the native protein, we propose that processing may be necessary to expose these sites, perhaps for interaction with the cell membrane or the *Ia* of the antigen-presenting cell.**

The process leading to T-lymphocyte activation by antigen, fundamental to understanding T-cell function, differs from antibody recognition of antigen in several intriguing respects. Unlike antibodies, T cells do not generally distinguish native from denatured antigen (1–4) and must see antigen in conjunction with histocompatibility antigens on the surface of another cell, called a presenting cell (4–6). We provide evidence here, using a single clone of normal antigen-specific T cells and lysosomotropic agents or the protease inhibitor leupeptin, that an intact native antigen, myoglobin, requires a proteolytic lysosomal pathway for presentation, whereas a small antigenic fragment of myoglobin does not. Unexpectedly, a denatured form of the molecule, *S*-methylmyoglobin, behaves like the fragment, indicating that conformation as well as size is a determinant of processing requirements for T-cell activation. We suggest that this marked distinction between native myoglobin and fragment or denatured myoglobin reflects the effect of conformation on the ability of these molecules to interact with cell surface structures and explains why T cells, unlike antibodies, do not distinguish native from denatured antigen.

Previous studies have demonstrated that antigen presentation by macrophages as well as by B-tumor cells may be inhibited by agents that interfere with lysosomal function and cellular transport (chloroquine, NH_4Cl, and monensin) (7–10). In addition, ovalbumin that has been predigested with trypsin, but not urea-denatured, can be presented to ovalbumin-specific T-cell hybridomas by glutaraldehyde-fixed macrophages (11). All of these studies are consistent in suggesting that size is a critical factor and that large native antigens may require proteolysis prior to presentation. In the current study, we have taken advantage of a single T-cell clone (14.5) that responds equally well to the same antigenic determinant around Lys-140 (in association with *I-E*^{d}) when the determinant is presented on native sperm whale myoglobin, the CNBr-cleaved fragment 132–153, and the intact but conformationally altered protein in the form of either *S*-methylmyoglobin or apomyoglobin and have found that, at least for an antigen the size of myoglobin, conformation may be more important than size in determining whether processing of antigen is necessary for T-cell activation.

MATERIALS AND METHODS

T-Cell Clones. The T-cell clones were prepared from the lymph nodes of myoglobin-immune B10.D2 mice by the method of Kimoto and Fathman (12) as modified by Matis *et al.* (13). The clones were maintained in culture by alternating cycles of stimulation with antigen and 3000 rad-irradiated (1 rad = 0.01 J/kg) splenic presenting cells for 5 days followed by 10–14 days of rest in culture with fresh irradiated spleen cells but no antigen. The preparation of these clones has been described (14). Clone 14.5 is specific for an epitope of myoglobin involving Lys-140 and responds to the 132–153 fragment of myoglobin made by CNBr cleavage (15).

Antigens. Sperm whale myoglobin was repurified by the method of Hapner *et al.* (16) from a commercial preparation (Biozyme, South Wales, UK) and the major chromatographic component IV was used (17). Fragments were prepared as described (18, 19) by cleavage with CNBr. Apomyoglobin was prepared by titration in water to pH 1.5 with HCl and extraction of the heme with 2-butanone, followed by extensive dialysis. *S*-Methylmyoglobin was prepared by reaction of the methionines of apomyoglobin with methyl iodide as described (20, 21) and was dialyzed before use to ensure that it was free of any fragments, although none should have been generated in the reaction.

Inhibitors. Chloroquine (Sigma) and NH_4Cl (Baker) were made as stock solutions in RPMI medium and stored at

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−20°C until use. Monensin (Sigma) was dissolved in absolute ethanol, diluted in RPMI medium, and freshly made each time. Leupeptin (Boehringer Mannheim) was stored at −20°C and freshly dissolved in RPMI medium for each experiment.

Antigen-Presentation Assay. Normal B10.D2 mouse spleen cells were incubated with antigen, at the concentrations indicated, for 2 hr at 37°C, washed, irradiated with 2000 rads, and set up in microtiter wells of 96-well plates (Costar 3596; Cambridge, MA) with 1×10^4 cloned T cells (which had been rested without antigen for 10–16 days) in 0.2 ml of complete medium (a 1:1 mix of Eagle's/Hanks' amino acid and RPMI 1640 media with 10% fetal calf serum, 2 mM glutamine, 0.05 mM 2-mercaptoethanol, 100 μg of streptomycin per ml, and 100 units of penicillin per ml) without additional antigen. These were cultured for 4 days at 37°C in 6% CO_2 in air. On the fourth day, 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine was added per well, and, after further culture overnight, the cells were harvested on glass fiber filters and the incorporation of ^{3}H into DNA was measured by scintillation spectroscopy. The effect of various inhibitors on antigen processing or presentation was tested by pretreating the spleen cells with inhibitor before exposure to antigen and/or adding inhibitor during the culture with antigen, as indicated. In all cases, the inhibitors were washed out, along with antigen, before the irradiated presenting cells were cultured with the T-cell clones.

RESULTS AND DISCUSSION

Our initial observation was that splenic cells that were treated with chloroquine prior to a 2-hr antigen exposure could present fragment 132–153 but not native myoglobin to the T-cell clone (Fig. 1). Treatment of presenting cells with NH_4Cl or monensin during antigen exposure produced similar results (Fig. 2). Chloroquine and NH_4Cl are weak bases that accumulate in lysosomes, raise lysosomal pH, and interfere with lysosomal movement as well as trapping recirculating receptors (22, 23). Monensin is a cationic ionophore that inhibits intracellular movement of lysosomes, lysosomal products, and newly synthesized proteins through the Golgi and possibly raises lysosomal pH (24). These results demonstrate that a lysosomal pathway is required for the presentation of native myoglobin but not that of the fragment.

Since fragment 132–153 differs from native myoglobin in size as well as conformation, we used *S*-methylmyoglobin to see which of these alterations was responsible for the difference in requirements for presentation. *S*-Methylmyoglobin is similar in size to the native molecule but has been partially denatured and unfolded by the addition of a second methyl group to hydrophobic methionine residues at positions 55 and 131, thereby introducing positive charges into the hydrophobic core of the molecule (20, 21). These require the molecule to unfold to admit solvent. NMR studies of [^{13}C]methyl groups introduced into the methionine residues, showing narrow resonances with equivalent chemical shifts identical to free *S*-methylmethionine, demonstrate that both residues are freely exposed to solvent and in an equivalent environment to one another, whereas in the native molecule, the two methionine residues are in very different environments inside the protein and not freely exposed to solvent, as shown by very different chemical shifts and line broadening in NMR (20, 21). Surprisingly, this form of denatured myoglobin stimulated clone 14.5 under conditions of chloroquine treatment that inhibited presentation of native myoglobin (Fig. 3). We conclude that *S*-methylmyoglobin does not require a lysosomal pathway for processing and that conformational change alone may be sufficient for presentation.

To investigate in a more specific manner the role of proteases in processing, we used leupeptin, a tripeptide (acetyl-Leu-Leu-Arg) competitive protease inhibitor that selectively inhibits protein degradation in lysosomes (25, 26). We studied presentation of native myoglobin, fragment 132–153, and *S*-methylmyoglobin in the presence or absence of leupeptin during antigen exposure of presenting cells (Fig. 4). In addition, we studied a second intact but structurally altered form of myoglobin, apomyoglobin. The removal of the heme in the preparation of apomyoglobin deprives the molecule of an important internal stabilizing component and produces a more flexible polypeptide structure relative to native myoglobin (27–29) (see below). The presentation of native myoglobin was inhibited by leupeptin, whereas that of fragment, of apomyoglobin, and of *S*-methylmyoglobin was not inhibited. The mechanism of inhibition by leupeptin is consistent with competitive inhibition since high concentrations of native myoglobin (12 μM) will overcome the inhibition by leupeptin but not by NH_4Cl (results not shown). Although competitive inhibition by leupeptin is convincing evidence for proteolysis, secondary effects on lysosomal function cannot be entirely excluded. The leupeptin alone, on antigen-presenting cells, does not stimulate T cells. The results shown in Fig. 4 also confirm the importance of conformation rather than size in determining the need for proteolytic processing. Thus, proteolysis may be primarily required to unfold the native conformation (see below). Although each of the inhibitors used may have multiple and potentially toxic effects on cellular function, since we demonstrate a difference in processing requirements dependent only on antigen structure, it is unlikely that these treatments have interfered with general

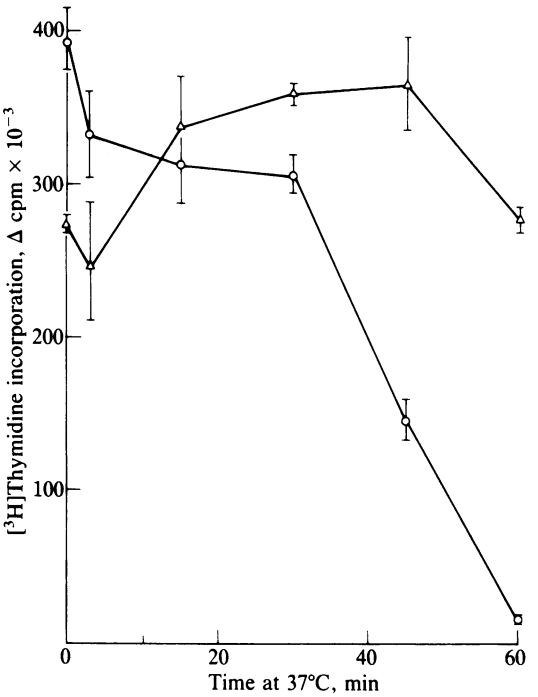

FIG. 1. Chloroquine pretreatment of presenting cells inhibits T-lymphocyte activation of native antigen but not fragment 132–153. Syngeneic spleen cells were incubated with 300 μM chloroquine at 37°C for the times indicated, washed, incubated with 2 μM myoglobin or 1 μM fragment 132–153 for 2 hr at 37°C, washed, irradiated, and placed into culture at 500,000 cells per well with clone 14.5 at 10,000 cells per well. The responding T cells, clone 14.5, had been cloned by limiting dilution and maintained in culture by alternate stimulation with antigen (sperm whale myoglobin) and rest without antigen. For assay of stimulation, cells that were rested for 14 days were placed in 96-well microtiter plates. On the fourth day, 1 μCi of [^{3}H]thymidine was added and the incorporation of ^{3}H into DNA was measured after further culture overnight. △, Fragment 132–153; ○, native myoglobin.

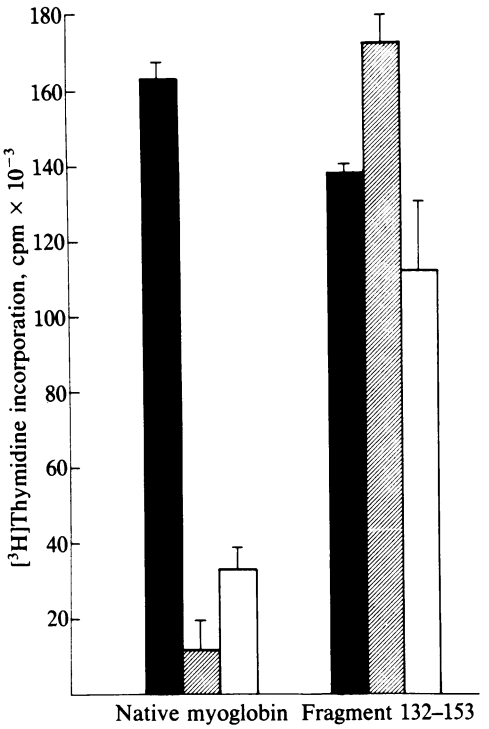

FIG. 2. Differential inhibition of antigen presentation by monensin or ammonium chloride. Syngeneic spleen cells were incubated for 15 min at 37°C prior to and during a 2-hr antigen exposure with either 10 mM NH_4Cl (▨) or 30 mM monensin (□), washed, counted, irradiated, placed into culture at 500,000 cells per well with 10^4 T cells of clone 14.5, and cultured as described in the legend to Fig. 1. ■, No inhibitor. Data are expressed as mean ± SEM for triplicate cultures.

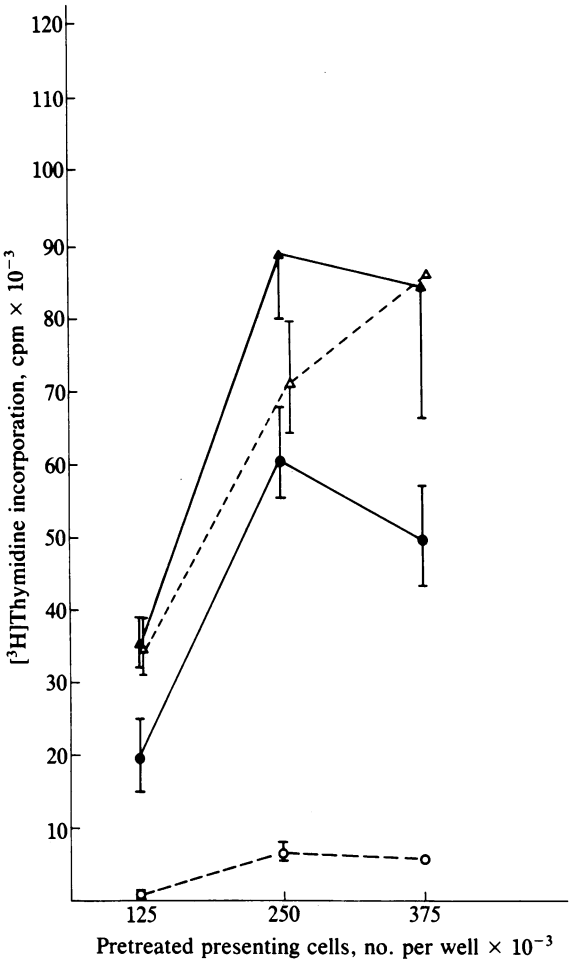

FIG. 3. Effect of conformation on chloroquine inhibition of antigen presentation. Syngeneic spleen cells were incubated at 37°C with or without 600 μM chloroquine for 45 min, washed, incubated with either 2 μM sperm whale myoglobin or 1 μM *S*-methylmyoglobin, and placed into culture at the number of cells indicated on the abscissa (×10^3), as described in the legend to Fig. 1. △, Chloroquine, then *S*-methylmyoglobin; ▲, *S*-methylmyoglobin; ●, native myoglobin; ○, chloroquine, then native myoglobin.

cellular function or with a specific step that is a general feature of antigen presentation other than the alteration of antigen to a form that is recognized by the T-cell receptor and able to interact with *Ia*.

S-Methylmyoglobin and apomyoglobin do not differ in size from native myoglobin, yet have significantly different processing requirements compared to the native form. One explanation for this difference may be that unfolded antigen is more susceptible to proteolysis than native sperm whale myoglobin. However, the failure to detect inhibition in our assay system over a wide range of concentrations of inhibitor, antigen, and presenting cells suggests that such proteolysis, if it were to occur, would involve a different pathway from that used to process the native protein.

What could be the critical difference in conformation between native and *S*-methylmyoglobin that can obviate a processing step? What property is common to native, water-soluble globular proteins that distinguishes them from denatured forms? We suggest that the hydrophobic regions that are buried in the interior of the native globular protein are more accessible when the molecule is opened up as it is in *S*-methylmyoglobin by the placement of positive charges in the core of the molecule. Similarly, the fragment cannot bury its hydrophobic residues as well as the native protein. Apomyoglobin, although less denatured than *S*-methylmyoglobin, has 15–20% less α-helical content and is more flexible because of the removal of the heme that bridges several helices (28, 29). The greater flexibility of apomyoglobin compared to native myoglobin was also demonstrated by the accessibility to specific modifying reagents of residues not accessible in the native form (27–29). The energy required to unfold apomyoglobin must therefore be considerably less than that of the native form, as witnessed by the greater ease of denaturation. Therefore, for all three altered forms of the antigen, the conformational changes necessary to allow interaction with the hydrophobic cell membrane of the presenting cell would require considerably less energy than that required in the case of the native molecule. It has been suggested that in addition to the site on the antigen that is bound by the T-cell receptor, a second site on the antigen is necessary for binding to *Ia* histocompatibility antigens (6, 13, 30). Perhaps this *Ia*-binding site on the antigen must be hydrophobic to interact with hydrophobic binding sites on *Ia* molecules for presentation or perhaps hydrophobic sites are necessary for interaction with other cell membrane components. An essential feature of antigen processing would thus be exposure of critical hydrophobic sites of globular proteins.

Alternatively, it is possible that processing is necessary to expose some other type of site, such as one interacting with the T-cell receptor. We think this alternative to be less likely because, in analogy with antibodies, the T-cell receptor probably interacts with hydrophilic sites already exposed on the surface of the protein. Indeed, the hydrophilic residue Lys-140 has already been identified as a major component of the determinant recognized by this T-cell clone 14.5 (15), and for the other group of T-cell clones we have characterized, all are specific for a determinant involving the hydrophilic residue Glu-109 (14). In any case, the function of proteolytic processing would be to expose critical sites on the antigen, not merely to reduce its size.

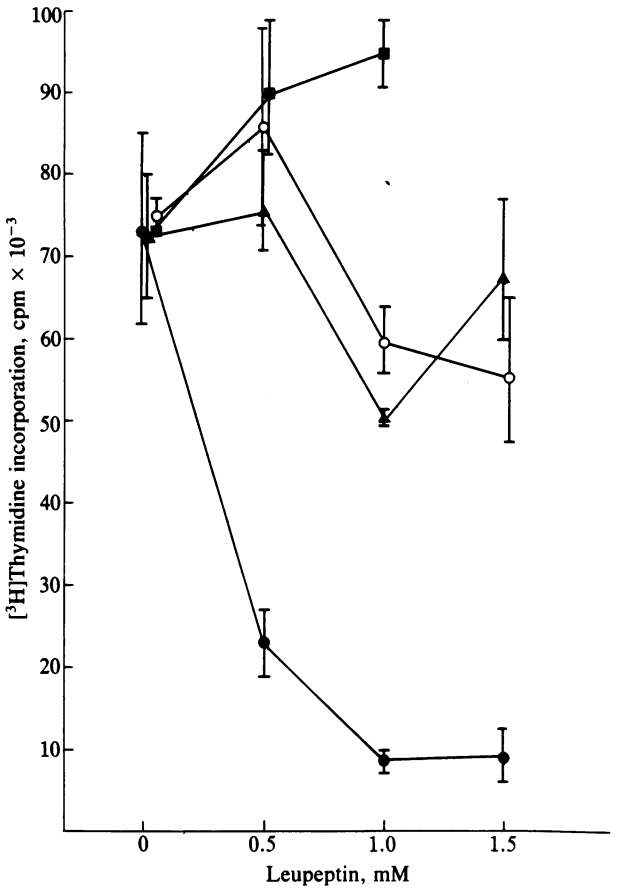

FIG. 4. Effect of size and conformation on inhibition of antigen presentation by leupeptin. Syngeneic spleen cells were incubated with leupeptin at the concentrations indicated, for 15 min prior to and during exposure to 2 μM native myoglobin (●), 1 μM apomyoglobin (▲), 1 μM *S*-methylmyoglobin (■), or 1 μM fragment 132–153 (○), washed, counted, irradiated, and placed into culture at 200,000 cells per well, as described in the legend to Fig. 1, with 10^4 cells of clone 14.5 per well.

If this explanation is correct we would expect that not all denatured forms of antigen would be sufficient for presentation without proteolysis. Therefore, we see no inconsistency between our results and hypothesis and the results of a recent study (11) using a very different system involving urea-denatured, reduced, and carboxymethylated ovalbumin in comparison with native and fragmented ovalbumin for presentation by glutaraldehyde-fixed B-lymphoma cells to T-cell hybridoma tumor lines. In this system, only antigen fragments, but not the native or denatured antigen, were presented by the glutaraldehyde-fixed cells. Since the authors indicate that the denatured ovalbumin was largely aggregated, it is reasonable to suppose that this form of denaturation does not open up the molecule in a way that exposes critical sites.

We have established a relationship between antigen conformation as well as size and the requirement for processing prior to T-lymphocyte recognition. A small 22-residue fragment containing the antigenic site does not require the same processing as the native molecule. Moreover, at least for an antigen the size of myoglobin (M_r 17,800), appropriately unfolded or more flexible intact molecules appear similar to fragment in having minimal processing requirements. On the one hand, denaturation may increase susceptibility to proteolysis or allow processing via pathways not accessible to the native molecule, but, on the other hand, proteolysis of native protein may merely be one method of inducing denaturation. It may be this type of denaturation resulting in increased flexibility and exposure of critical sites necessary for interaction with cell membrane or histocompatibility antigens, rather than size, which is critical for T-cell recognition. Of course, since our study concerns a protein that is already relatively small in the native form, it is quite possible that for a larger protein, proteolysis would be important for reduction of size as well as for alteration of conformation.

These results may explain the age-old dilemma that T cells generally do not distinguish native from denatured antigen, since our results indicate that antigen must first be unfolded by some mechanism, such as, but not limited to, proteolysis, before it can be presented to T lymphocytes. In fact, this phenomenon may reflect the second intriguing property of T-lymphocyte recognition—namely, the need to see antigen in association with histocompatibility antigens on a cell membrane. These results may also have implications for the role of antigen denaturation in the induction of autoimmune disease, such as systemic lupus, autoimmune thyroiditis, and insulin-dependent diabetes mellitus, as well as for our understanding of the complex mechanism by which T lymphocytes, in contrast to antibodies, recognize antigen.

Note Added in Proof. After this manuscript was submitted for publication, it came to our attention that Allen and Unanue and coworkers (31, 32), using a different approach and studying T cells specific for hen lysozyme, have also just concluded that hydrophobic sites are likely to be important in antigen presentation. This study and ours thus independently support one another.

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1. Gell, P. G. H. & Benacerraf, B. (1959) *Immunology* **2**, 64–70.
2. Ishizaka, K., Kishimoto, T., Delespesse, G. & King, T. P. (1974) *J. Immunol.* **113**, 70–77.
3. Schirrmacher, V. & Wigzell, H. (1974) *J. Immunol.* **113**, 1635–1643.
4. Berzofsky, J. (1980) in *Biological Regulation and Development*, ed. Goldberger, R. F. (Plenum, New York), Vol. 2, pp. 467–594.
5. Rosenthal, A. S. (1978) *Immunol. Rev.* **40**, 136–156.
6. Benacerraf, B. (1978) *J. Immunol.* **120**, 1809–1812.
7. Ziegler, H. K. & Unanue, E. R. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 175–178.
8. Chesnut, R. W., Colon, S. M. & Grey, H. M. (1982) *J. Immunol.* **129**, 2383–2387.
9. Lee, K. C., Wong, M. & Spitzer, D. (1982) *Transplantation* **34**, 150–152.
10. Scalla, G. & Oppenheim, J. J. (1983) *J. Immunol.* **131**, 1160–1165.
11. Shimonkevitz, R., Kappler, J., Marrack, P. & Grey, H. (1983) *J. Exp. Med.* **158**, 303–316.
12. Kimoto, M. & Fathman, C. G. (1980) *J. Exp. Med.* **152**, 759–770.
13. Matis, L. A., Longo, D. L., Hedrick, S. M., Hannum, C., Margoliash, E. & Schwartz, R. H. (1983) *J. Immunol.* **130**, 1527–1535.
14. Berkower, I. J., Matis, L. A., Buckenmeyer, G. K., Gurd, F. R. N., Longo, D. L. & Berzofsky, J. A. (1984) *J. Immunol.* **132**, 1370–1378.
15. Berkower, I. J., Matis, L. A., Longo, D. L., Gurd, F. R. N. & Berzofsky, J. A. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **42**, 1242 (abstr.).
16. Hapner, K. D., Bradshaw, R. A., Hartzell, C. R. & Gurd, F. R. N. (1968) *J. Biol. Chem.* **243**, 683–689.
17. Berzofsky, J. A. (1978) *J. Immunol.* **120**, 360–369.
18. Marshall, R. C., Jones, W. C., Jr., Vigna, R. A. & Gurd, F. R. N. (1974) *Z. Naturforsch., C* **29**, 90–91.
19. Berzofsky, J. A., Richman, L. K. & Killion, D. J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4046–4050.
20. Jones, W. C., Jr., Rothgeb, T. M. & Gurd, F. R. N. (1975) *J. Am. Chem. Soc.* **97**, 3875–3877.

21. Jones, W. C., Jr., Rothgeb, T. M. & Gurd, F. R. N. (1976) *J. Biol. Chem.* **251,** 7452–7460.
22. Ohkuma, S. & Poole, B. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 3327–3331.
23. Hopgood, M. F., Clark, M. G. & Ballard, F. J. (1977) *Biochem. J.* **164,** 399–407.
24. Basu, S. K., Goldstein, J. L., Anderson, R. G. W. & Brown, M. S. (1981) *Cell* **24,** 493–502.
25. Grinde, B. & Seglen, P. O. (1980) *Biochim. Biophys. Acta* **632,** 73–86.
26. Aoyagi, T. & Umezawa, H. (1981) *Acta Biol. Med. Ger.* **40,** 1523–1529.
27. Breslow, E. (1964) *J. Biol. Chem.* **239,** 486–496.
28. Breslow, E., Beychok, S., Hardman, K. D. & Gurd, F. R. N. (1965) *J. Biol. Chem.* **240,** 304–309.
29. Harrison, S. C. & Blout, E. R. (1965) *J. Biol. Chem.* **240,** 299–303.
30. Heber-Katz, E., Hansburg, D. & Schwartz, R. H. (1983) *J. Mol. Cell Immunol.* **1,** 3–14.
31. Allen, P. M. & Unanue, E. R. (1984) *J. Immunol.* **132,** 1077–1079.
32. Allen, P. M., Strydom, D. J. & Unanue, E. R. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2489–2493.