Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 5220–5223, July 1988
Immunology

Direct evidence for functional self-protein/Ia-molecule complexes *in vivo*

(autoimmunity/antigen presentation/hemoglobin/T cell)

ROBIN G. LORENZ AND PAUL M. ALLEN

Department of Pathology, Washington University School of Medicine, 660 South Euclid Avenue, Saint Louis, MO 63110

Communicated by Emil R. Unanue, April 15, 1988

ABSTRACT **Through the development of a panel of murine hybridomas reactive to murine hemoglobin, we have been able to study the processing and presentation of self antigens by antigen-presenting cells. Our results demonstrate that peritoneal macrophages *in vivo* can process and potentially present the self-antigen hemoglobin. We have extended this finding to show that, directly after removal from the mouse, antigen-presenting cells from a variety of tissues stimulate our hemoglobin-specific hybridomas without any manipulation or addition of exogenous antigen. This constitutes direct functional proof that in a nondisease state self proteins are processed constitutively and can be presented in a fashion similar to that in which foreign antigens are presented. Our demonstration that antigen-presenting cells can process and potentially present self as well as foreign molecules implies that self-tolerance occurs at the level of the T cell. This constitutive processing and presentation of self antigens has potentially far-reaching implications in self-tolerance, autoimmunity, and alloreactivity.**

The ability to discriminate self from nonself is a hallmark of the immune response. However, the mechanisms involved in this discrimination are poorly understood (1, 2). Discrimination could be functioning at the level of the T cell or the antigen-presenting cell (APC). T cells recognize foreign protein antigens in the context of self-histocompatibility molecules (3, 4), usually after the antigen has undergone processing by an APC (5). However, the question remains whether this APC *in vivo* can distinguish foreign molecules from self molecules (6). To determine if an APC distinguishes between self and foreign molecules, we have generated murine T-cell hybridomas specific for murine hemoglobin (Hb). These hybridomas were used as probes to detect the presence or absence of processed murine Hb on the surface of murine APCs.

Murine Hb is a four-chain molecule composed of two $\alpha\beta$ dimers. Hb circulates enclosed in erythrocytes and is degraded predominantly within the macrophages (Mϕ) of the reticuloendothelial system after phagocytosis of senescent erythrocytes. Allelic variants of both the α and β chains are found in inbred mouse strains. To directly examine for the presence of self-antigen/Ia-molecule complexes we utilized two *H-2*^{k} mouse strains (CBA/J and CE/J) that express different alleles of Hb. We generated Ia^k-restricted T-cell hybrids specific for one allele of Hb by immunizing one strain with Hb from the opposite strain. These T-cell hybrids were then used as probes to detect these complexes and were found to be triggered by APCs from tissues of mice that expressed the immunizing Hb antigen.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Mice. CBA/J and CE/J 5- to 8-week-old mice of either sex were obtained from The Jackson Laboratory. Residential Mϕ APCs were obtained from female CBA/J and CE/J retired breeder mice.

Antigen Preparation. Hemolysate was prepared from CBA/J blood obtained via retro-orbital puncture. The erythrocytes were pelleted (3000 × *g*, 10 min) and washed three times with normal saline. The cells were lysed with 2 vol of distilled water and the membranes were pelleted out by centrifugation at 10,000 × *g* for 20 min. The Hb concentration was determined by the absorbance at 280 nm. The hemolysate was approximately 95% Hb. The peptide Hb$^{\beta dmin}$-(67–76) was synthesized by using a DuPont RaMPs multiple peptide synthesis system, and the composition was verified by fast atom bombardment mass spectrometry.

Development of Hb-Specific T-Cell Hybridomas. Hb-specific hybridomas were generated by using a standard fusion protocol (7). Briefly, CE/J (*Hbb*^{s}) popliteal lymph nodes were taken 10 days after immunization in the hind footpads with 20 μg of CBA/J (*Hbb*^{d}) Hb emulsified in complete Freund's adjuvant (Difco H37Ra). A single-cell suspension was prepared and stimulated *in vitro* with Hb at 100 μg/ml for 4 days. These bulk cultures were harvested and fused with the BW5147 fusion partner. All hybridomas used were subcloned twice by limiting dilution analysis to ensure clonality of the line.

T-Cell Hybridoma Stimulation Assay. The assay system examines the *in vivo* presentation of Hb by APCs to the Hb-specific T-cell hybridomas YO1.6 and WK5.1. APCs used were the B-cell hybridoma TA3 or residential peritoneal Mϕ. The peritoneal cells were allowed to adhere for 2 hr in 96-well microtiter plates. The monolayer formed was washed to remove nonadherent cells and the adherent cells either were used directly (live) or were fixed with 1% paraformaldehyde (fixed) (8). To this monolayer was added the T-cell hybridoma (10^5 cells per well). No exogenous antigen was added. T-cell stimulation was measured by quantitating the release of interleukin 2 by the T-cell hybridoma. Interleukin 2 was quantitated by measuring [^{3}H]thymidine incorporation by the interleukin 2-dependent CTLL line (8). The TA3 cells were used in suspension either untreated (live) or fixed with 1% paraformaldehyde (fixed). To these presenting cells (3 × 10^4 per well) was added the antigen together with the YO1.6 hybridoma.

APC Preparation. Mice were perfused through the heart with 40 ml of sterile Dulbecco's phosphate-buffered saline

Abbreviations: APC, antigen-presenting cell; Mϕ, macrophages; Hb, hemoglobin; *Hbb*, hemoglobin allele expressed at the hemoglobin β-chain locus; Hb$^\beta$, hemoglobin β-chain protein; RP-HPLC, reverse-phase high-performance liquid chromatography; HEL, hen egg-white lysozyme; mAb, monoclonal antibody; MHC, major histocompatibility complex.

(GIBCO) to remove erythrocytes from the tissues. The organs were rapidly removed and a single-cell suspension was prepared. The tissues were kept at 0°C to prevent antigen processing during the preparation. The cells were washed and cultured in a 96-well microtiter plate with 1×10^5 hybridoma cells per well. B cells were obtained from spleen cells by a series of purification steps. A single-cell suspension of spleen cells was prepared, then separated on a three-step Percoll (Pharmacia) gradient and the small B-cell population was taken. These small B cells were treated sequentially with anti-Thy-1.2 (AT83A) and anti-L3T4 (GK1.5), then guinea pig complement to remove any remaining T cells. This B-cell population was then labeled with rabbit anti-mouse surface immunoglobulin and fluorescein isothiocyanate-labeled goat antibodies to rabbit immunoglobulin, washed, sorted by a fluorescence-activated cell sorter (FACS), and then used as APCs. B cells were not prepared from CE/J mice. Alveolar Mϕ were obtained by alveolar lavage as described (9). Peripheral blood mononuclear cells were obtained from blood collected into EDTA. The mononuclear cells were separated from the erythrocytes by using a Ficoll–Hypaque (Pharmacia) gradient.

Analysis of Tryptic Fragments of Hb. A tryptic digest of CBA/J hemolysate was separated into distinct fragments by reverse-phase high-performance liquid chromatography (RP-HPLC). Each fraction was tested for stimulation of the Hb-specific hybridomas. The positive peak was purified further by RP-HPLC and identified by amino acid analysis using a Waters Pico-Tag amino acid analyzer. The composition of this stimulatory fragment was confirmed by fast atom bombardment mass spectrometry.

Hen Egg-White Lysozyme (HEL)-Specific T-Cell Hybridomas. T-cell hybridomas specific for HEL have been described previously (8). The hybrid used in this study is the 3A9 T-cell, which is specific for I-A^k/HEL-(46–61).

Monoclonal Antibodies (mAbs). The hybridomas used in this study were classified as I-E^k- or I-A^k-restricted on the basis of blocking studies with the I-$E^{k/d}$-specific 14-4-4s antibody and the I-A^k-specific 10–2.16 antibody. These mAbs were obtained from the American Type Culture Collection (ATCC) and have been described previously (10, 11). The anti-Thy-1.2 (AT83A) mAb and the anti-L3T4 (GK1.5) mAb were also obtained from the ATCC.

RESULTS AND DISCUSSIONS

The T-cell hybridomas used in this study were generated by immunizing CE/J mice (*Hbb^s*) with *Hbb^d* Hb. $Hb^{\beta d}$ has numerous amino acid differences from $Hb^{\beta s}$ (Fig. 1 *Upper*). Of the panel of 58 Hb-specific hybridomas characterized, 54 were I-E^k-restricted and 4 were I-A^k-restricted, as determined by antibody blocking studies (data not shown). Hemolysate of the *Hbb^d* haplotype stimulated the hybridoma panel, whereas *Hbb^s* hemolysate did not, indicating that the hybrids were Hb-allele specific. All of the T cells had conventional antigen reactivity and were not auto-Ia reactive in that they could be stimulated only by the addition of Hb to TA3 cells, a B-cell hybridoma that served as an APC (data not shown). None responded to TA3 cells alone or to Hb in the absence of an APC. Two hybrids were chosen to represent the panel: YO1.6, an I-E^k-restricted hybrid, and WK5.1, which is I-A^k-restricted.

It has been shown that fixed APCs cannot present the native forms of globular antigens but can present processed forms of the same antigen (8, 15). YO1.6 required processing of Hb, since the fixed TA3 cells could not present it (Fig. 2 *Lower*). Similar results have obtained with WK5.1 (data not shown). Therefore, YO1.6 and WK5.1 are stimulated only by Hb processed by an APC. The portion of Hb specifically recognized by our hybridomas was determined by screening

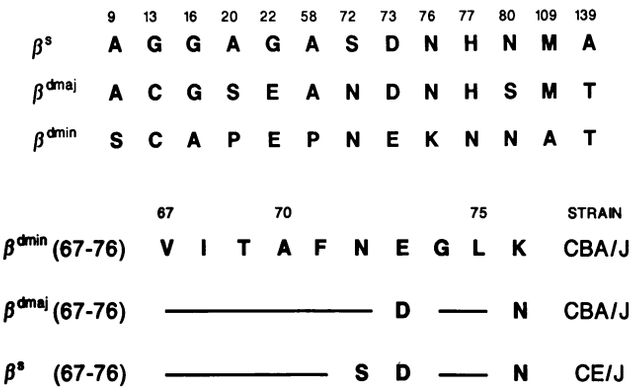

FIG. 1. (*Upper*) Amino acid substitutions in the mouse β-globins (12). Numbers shown above the amino acid symbols designate the residue position in native β-globin. Amino acids are identified by the single-letter code. Only the amino acid positions where the alleles of *Hbb* vary are shown. (*Lower*) Primary amino acid sequence of Hb^{β}-(67–76). CBA/J mice (*Hbb^d*) produce two variant β-chains in unequal amounts (13): 80% of the total β chain is β^{dmaj} and 20% is β^{dmin}. CE/J mice (*Hbb^s*) produce a single β chain (14).

a panel of peptides generated by trypsin digestion of denatured Hb. Both YO1.6 and WK5.1 responded to the peptide $Hb^{\beta dmin}$-(67–76), either purified from the tryptic digest or as a synthetic peptide (Fig. 2 *Upper* and data not shown). The equivalent synthetic peptide Hb-(67–76) from $Hb^{\beta dmaj}$ (Fig. 1 *Lower*) was about 1/300th to 1/1000th as active and, as expected, the peptide corresponding to the $Hb^{\beta s}$-(67–76) sequence was nonstimulatory (data not shown). Thus, both the I-A^k-restricted T cell WO5.1 and the I-E^k-restricted T cell YO1.6 recognized the same 10-residue peptide, Hb-(67–76), of the $Hb^{\beta dmin}$ polypeptide chain.

These murine Hb-specific, Ia^k-restricted hybridomas were then used as probes to determine if APCs constitutively expressed self-Hb/Ia complexes. Two *H-2^k* strains were used as sources of APCs, CBA/J (*Hbb^d*), which produces *in vivo* the Hb for which our hybrids are specific, and CE/J (*Hbb^s*),

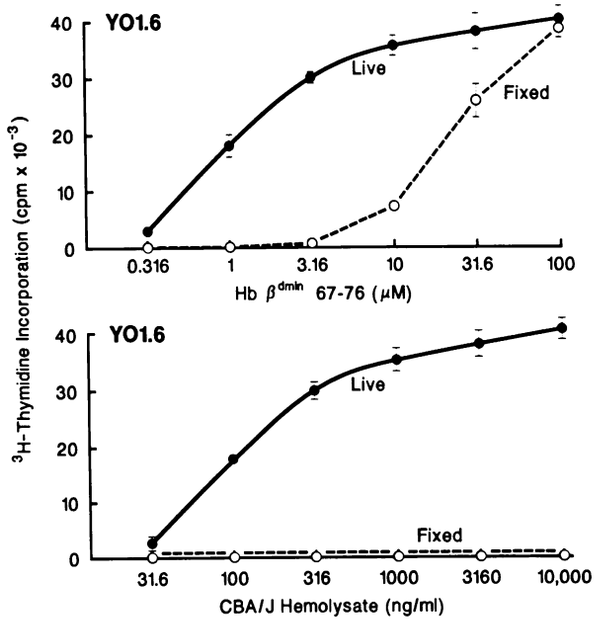

FIG. 2. Stimulation of the Hb-specific I-E^k-restricted hybridoma YO1.6 by peptide $Hb^{\beta dmin}$-(67–76) (*Upper*) or by intact Hb (*Lower*). APCs used were the B-cell hybridoma TA3. The stimulation assay was performed as described in the text. The TA3 cells were used in suspension at 3×10^4 per well either untreated (live) or fixed with 1% paraformaldehyde (fixed). Error bars represent ±SD.

which produces the nonstimulating Hb molecule. Residential peritoneal Mϕ harvested from normal CBA/J mice were able to stimulate both Hb-specific hybridomas without addition of exogenous antigen (Fig. 3), providing direct functional evidence that Hb/Ia complexes are present *in vivo* on the surface of these APCs. Since Hb needs to be processed prior to presentation (Fig. 2), these results demonstrate that APCs must be normally processing and potentially presenting self-antigens. This formation of Ia/self-antigen complexes was not limited to a single Ia molecule, since both Hb/I-A^k and Hb/I-E^k complexes were detected by WK5.1 and YO1.6, respectively.

Our functional assay using peritoneal Mϕ involves a 2-hr adherence step. To eliminate the possibility that the complexes we were detecting were only generated *in vitro* during this 2-hr period by processing of self-Hb (either in erythrocytes or in some other form), two experiments were performed. First, residential peritoneal Mϕ were continuously treated with chloroquine (0.1 mM), a lysosomotropic amine, during the 2-hr adherence step. It has been well established that treatment of APCs with chloroquine completely inhibits antigen processing (16). These treated Mϕ were still able to stimulate YO1.6 without the addition of exogenous antigen, whereas the presentation of the control antigen HEL to 3A9 T cells was completely abrogated (data not shown) (8). Second, spleen cells were depleted of erythrocytes by centrifugation on Ficoll-Hypaque at 0°C, and the mononuclear cells were immediately fixed. These cells were also able to stimulate YO1.6 (data not shown). These two experiments eliminate the possibility that Hb was being processed and presented only *in vitro*. It is conceivable that the ability of CBA/J Mϕ to stimulate the Hb-specific hybridomas reflects the recognition of a crossreactive determinant, other than Hb, on the surface of the CBA/J APC (17). This potential antigen would arise from a polymorphic locus that differs between the CE/J and CBA/J inbred strains. We feel that this possibility is unlikely, as APCs from two additional *H-2*^{k}, *Hbb*^{d} inbred strains (C3H/HeJ and A/J) also stimulate the Hb-specific hybridomas and APCs from an *H-2*^{k}, *Hbb*^{s} inbred strain (B10.BR/SgSnJ) did not stimulate the hybridomas (data not shown).

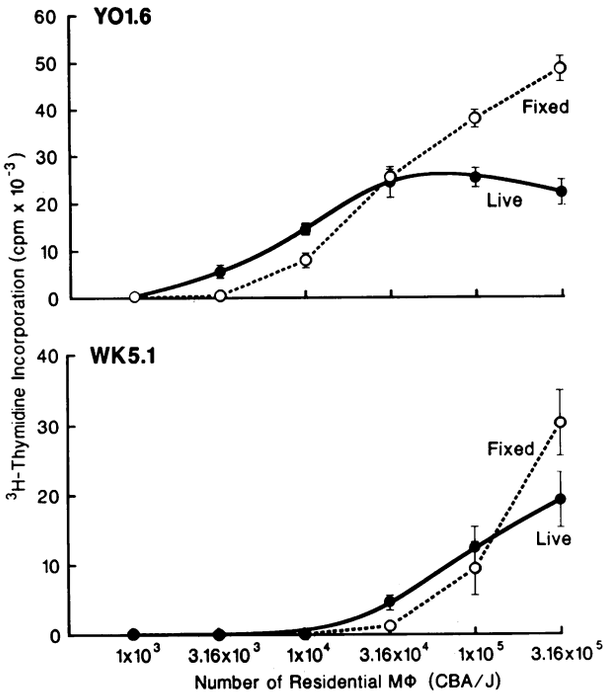

FIG. 3. Stimulation of the Hb-specific I-E^k-restricted YO1.6 (*Upper*) and I-A^k-restricted WK5.1 (*Lower*) by residential CBA/J peritoneal Mϕ. The stimulation assay was performed as described for Fig. 2 except that residential peritoneal cells were used as the APCs. No exogenous antigen was added.

This *in vivo* presentation of self antigens was not restricted to peritoneal Mϕ but was a widespread phenomenon with functional self-Hb/Ia complexes being found on the surface of tissue APCs in most major organs of the body (Fig. 4). These include thymus, lymph node, spleen, and lung. These self-antigen/Ia complexes are not limited to phagocytic cells, since purified splenic B cells also stimulated our hybridomas. Other tissues containing APCs that stimulate and that, therefore, must constitutively have Hb/Ia complexes on their surfaces include kidney, liver, brain, and heart (data not shown). We did not identify the Ia^+ presenting cells in these various tissues. Of the tissues we examined, only the pancreas did not have endogenous complexes, and no complexes were formed even after addition of exogenous Hb, indicating that there were not sufficient Ia^+ cells in our pancreatic preparation to be detected by our assay. Organs obtained from the nonstimulatory CE/J strain (*Hbb*^{s}) did not have constitutive Hb/Ia complexes on their surfaces that could stimulate YO1.6 or WK5.1 but they could form stimulatory complexes when exogenous Hb^{Bd} was added. As these results indicate that Hb/Ia complexes are found throughout the tissues, including those that are presumed not to be actively degrading erythrocytes, and even on nonphagocytic cells such as B-cells, we believe that there must be some mechanism for exposure to Hb or its fragments other than phagocytosing erythrocytes. A possible explanation is that soluble immunogenic peptides have been found to be released by Mϕ (i.e., exocytosed) (18).

There have been several reports of Ia^+ cells processing and presenting self antigens *in vitro*. For example, Winchester *et al.* (19) showed that APCs were equally able to present autologous and foreign F protein *in vitro* and Bogen *et al.* (20) reported that syngeneic immunoglobulin fragments were presented in the context of class II molecules by APCs *in vitro*. It has also been demonstrated that the peripheral

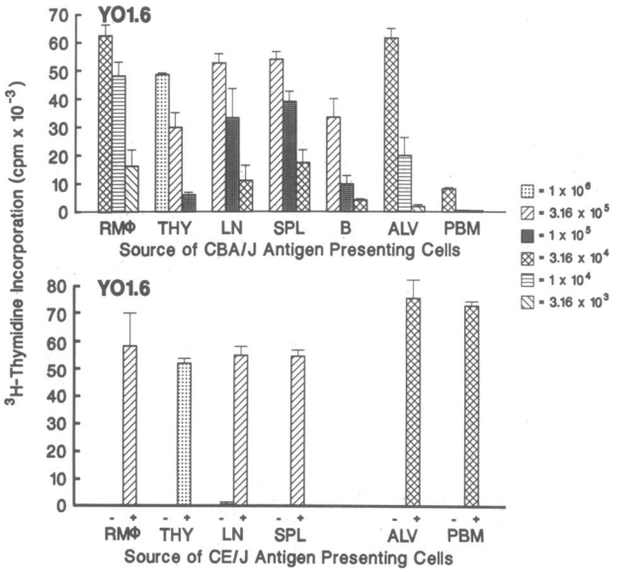

FIG. 4. Presentation by CBA/J (*Upper*) and CE/J (*Lower*) tissues to the Hb-specific YO1.6 hybridoma. No exogenous antigen was added to the CBA/J cell cultures (*Upper*). Hemolysate (*Hbb*^{d}) was present at 100 μg/ml (+) or absent (−) in *Lower*. The assay system is as described for Fig. 2. Mϕ were obtained by peritoneal lavage. RMϕ, resident peritoneal Mϕ; THY, thymus; LN, lymph node; SPL, spleen; B, splenic B cells; ALV, alveolar macrophages; PBM, peripheral blood mononuclear cells.

nervous system-derived Schwann cell can present myelin basic protein to T-cell lines in the absence of any exogenous antigen, but only after the APCs are activated by interferon γ (21). None of these reports have directly shown these self-antigen/Ia complexes to be expressed constitutively *in vivo*. Our demonstration of the constitutive presence of self-Hb/Ia complexes clearly indicates that APCs process and potentially present self as well as nonself molecules, and it raises the question of how we mount an immune response against foreign antigens in the presence of numerous self proteins. Further studies will be necessary to determine if APCs *in vivo* handle foreign molecules differently from self. One mechanism by which foreign antigens could overcome potential competition by self proteins is by enhanced uptake of the foreign molecule via a specific receptor on the APCs such as an immunoglobulin, fragment Fc, or complement receptor (22).

At this point, it is unknown how many other self proteins have the ability to form these complexes with Ia. The binding of self peptides to Ia has been demonstrated with mouse lysozyme and implicated with mouse cytochrome *c* (23, 24). However, the existence of formed complexes *in vivo* will depend on multiple factors in addition to ability to bind Ia, including the protein concentration and the intra- and extracellular location. In this respect Hb is an interesting molecule, as there is no circulating free Hb. All Hb is either inside erythrocytes, where it is found in very high concentrations, or bound to haptoglobin. Hb–haptoglobin complexes are cleared by the liver, while senescent erythrocytes are degraded primarily in the spleen, allowing a high local concentration of Hb to be obtained in these organs. Further studies will be necessary to determine if and where other self molecules form functional Ia/self-protein complexes. It has been shown in several systems that self-tolerance is restricted by the major histocompatibility complex (MHC) (25–28). CBA/J mice do not apparently react to these Hb/Ia complexes *in vivo*, implying that this tolerance has occurred via a functional or physical deletion of the reactive T cells. Our finding of these Hb/Ia complexes in the thymus leads us to speculate that these complexes could be directly involved in self-tolerance.

Our results directly prove that self antigens are processed and presented by normal APCs *in vivo*. The recent finding that alloreactive cytotoxic T lymphocytes can recognize HLA-derived peptides in the context of self-MHC molecules (29) leads to the speculation that self-MHC molecules are processed and presented in an analogous fashion to Hb, leading to alloreactivity toward self-MHC peptides in complex with another self-MHC molecule. Our results also imply that the unknown "peptide" found in the binding site of the recently crystallized class I MHC molecule may be self antigen (30).

We thank David Donermeyer and Barry Raynor for technical assistance and Marian Simandl and Jerri Smith for preparation of the manuscript. Mass spectrometry was performed by Dr. Andrew Tyler at the Washington University Mass Spectrometry Resource. This work was supported by Grants AI22033, AI24157, and GM07200 from the National Institutes of Health.

1. Nossal, G. J. V. (1983) *Annu. Rev. Immunol.* **1,** 33–62.
2. Zinkernagel, R. M. & Doherty, P. C. (1974) *Nature (London)* **248,** 701–702.
3. Rosenthal, A. S. & Shevach, E. M. (1973) *J. Exp. Med.* **138,** 1194–1212.
4. Rosenthal, A. S. (1978) *Immunol. Rev.* **40,** 136–152.
5. Unanue, E. R. (1984) *Ann. Rev. Immunol.* **2,** 395–428.
6. Raff, M. C. (1982) *Nature (London)* **298,** 791–792.
7. Allen, P. M. (1987) in *Monoclonal Antibody Production Techniques and Applications*, ed. Schook, L. B. (Dekker, New York), pp. 25–34.
8. Allen, P. M. & Unanue, E. R. (1984) *J. Immunol.* **132,** 1077–1079.
9. Weinberg, D. S. & Unanue, E. R. (1981) *J. Immunol.* **126,** 794–799.
10. Oi, V. T., Jones, P. P., Goding, J. W., Herzenberg, L. A. & Herzenberg, L A. (1978) *Curr. Top. Microbiol. Immunol.* **81,** 115–129.
11. Ozato, K., Mayer, N. & Sachs, D. H. (1980) *J. Immunol.* **124,** 533–540.
12. Russell, E. S. & McFarland, E. C. (1974) *Ann. NY Acad. Sci.* **241,** 25–38.
13. Gilman, J. G. (1976) *Biochem. J.* **159,** 43–53.
14. Whitney, J. B., Cobb, R. R., Popp, R. A. & O'Rourke, T. W. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 7646–7650.
15. Shimonkevitz, R., Kappler, J., Marrack, P. & Grey, H. M. (1983) *J. Exp. Med.* **158,** 303–316.
16. Ziegler, H. K. & Unanue, E. R. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 175–178.
17. Abromson-Leeman, S. R. & Cantor, H. (1983) *J. Exp. Med.* **158,** 428–437.
18. Allen, P. M., Beller, D. I., Braun, J. & Unanue, E. R. (1984) *J. Immunol.* **132,** 323–331.
19. Winchester, G., Sunshine, G. H., Nardi, N. & Mitchison, N. A. (1984) *Immunogenetics* **19,** 487–491.
20. Bogen, B., Malissen, B. & Haas, W. (1986) *Eur. J. Immunol.* **16,** 1373–1378.
21. Wekerle, H., Schwab, M., Linington, C. & Meyermann, R. (1986) *Eur. J. Immunol.* **16,** 1551–1557.
22. Rock, K. L., Benacerraf, B. & Abbas, A. K. (1984) *J. Exp. Med.* **160,** 1102–1113.
23. Babbitt, B. P., Matsueda, G., Haber, E., Unanue, E. R. & Allen, P. M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 4509–4513.
24. Lakey, E. K., Margoliash, E., Flouret, G. & Pierce, S. K. (1986) *Eur. J. Immunol.* **16,** 721–727.
25. Matzinger, P., Zamoyska, R. & Waldmann, H. (1984) *Nature (London)* **308,** 738–741.
26. Rammensee, H. G. & Bevan, M. J. (1984) *Nature (London)* **308,** 741–744.
27. Dos Reis, G. A. & Shevach, E. M. (1983) *J. Exp. Med.* **157,** 1287–1299.
28. Groves, E. & Singer, A. J. (1983) *J. Exp. Med.* **158,** 1483–1497.
29. Clayberger, C., Parham, P., Rothbard, J., Ludwig, D. S., Schoolnik, G. K. & Krensky, A. M. (1988) *Nature (London)* **330,** 763–765.
30. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L. & Wiley, D. C. (1987) *Nature (London)* **329,** 506–512.