Engl. J. Med. 295, 1149-1154.
Takano, M., Agris, P. F., & Sharp, G. C. (1980) *J. Clin. Invest. 65*, 1449-1456.
Tan, E. M., & Kunkel, H. G. (1966) *J. Immunol. 96*, 464-471.
Tan, E. M., & Rodnan, G. P. (1975) *Arthritis Rheum. 18*, 430.
Winn, D. M., Wolfe, J. F., Lindberg, D. A., Fristoe, F. H., Kingsland, L., & Sharp, G. C. (1979) *Arthritis Rheum. 22*, 1334-1337.
Wolfe, J. F., Adelstein, E., & Sharp, G. C. (1977) *J. Clin. Invest. 59*, 176-178.

Isolation and Sequence Analysis of the Intramembranous Hydrophobic Segment of the H-2K^b Murine Histocompatibility Antigen†

Hiroshi Uehara, John E. Coligan, and Stanley G. Nathenson*

ABSTRACT: The primary structure of the intramembranous segment (TC-1) of the mouse transplantation antigen, H-2K^b, has been determined. The segment contains a stretch of 31 uncharged amino acid residues and is localized between the NH_2-terminal and the COOH-terminal hydrophilic regions of the molecule. The amino acid sequence of TC-1 is Trp-Asp-Glu-Pro-Pro-Ser-Thr-Val-Ser-Asn-Met-Ala-Thr-Val-Ala-Val-Leu-Val-Val-Leu-Gly-Ala-Ala-Ile-Val-Thr-Gly-Ala-Val-Val-Ala-Phe-Val-Met-Lys-Met-Arg-(Arg)-(Arg).

The classical murine H-2 major histocompatibility antigens (H-2 antigens) are integral membrane glycoproteins which, although found on all cells, are more densely concentrated on lymphocytes. The molecules are composed of two noncovalently associated polypeptide chains, a highly polymorphic 45 000 molecular weight polypeptide coded for by genes within the major histocompatibility complex (MHC)[1] and a non-MHC-encoded 11 500 molecular weight polypeptide, β_2-microglobulin (β_2m) (Snell et al., 1976; Vitetta & Capra, 1979; Nathenson et al., 1981). Structural studies on the 45 000 molecular weight H-2K^b polypeptide have been pursued for the last 4 years by using radiochemical methodology (Nathenson et al., 1981; Coligan et al., 1981). The H-2K^b polypeptide was initially cleaved by CNBr, and three large fragments (>50 residues) and six small fragments (<35 residues) were isolated (Ewenstein et al., 1978). The amino acid sequence and alignment have been determined for the three large fragments and two of the smaller fragments, which comprise the NH_2-terminal 284 residues (Coligan et al., 1978; Uehara et al., 1980a,b; Martinko et al., 1980). As described in the following paper in this issue (Uehara et al., 1981), the four remaining CNBr fragments are found to constitute the COOH-terminal portion of the molecule (residues 308-346). However, the nine CNBr fragments which were recovered did not contain an extensive hydrophobic stretch which might interact with the lipid bilayer of the membrane. The presence of such a hydrophobic peptide would be expected, since it has been found in a number of integral membrane proteins such as cytochrome b_5 (Ozols & Gerard, 1977; Fleming et al., 1978; Takagaki et al., 1980), glycophorin A (Furthmayr et al., 1978), and membrane-bound IgM (Rogers et al., 1980). In addition, the total number of amino acid residues included in these CNBr fragments was smaller than the number predicted from the apparent molecular weight of the intact molecule. Thus, attempts were made to isolate a peptide containing the putative membrane-associated portion of the H-2K^b molecule employing procedures other than CNBr cleavage. In this paper, we report the isolation and amino acid sequence analysis of a tryptic peptide which contains the putative, intramembranous hydrophobic region of the H-2K^b molecule.

Materials and Methods

Radiolabeling, Cell Extraction, and Alloantisera. Incorporation of radiolabeled amino acids into EL-4.BrdU cells and cell extraction with the nonionic detergent Nonidet P-40 were performed as described previously (Uehara et al., 1980a; Nairn et al., 1980). Alloantisera against H-2K^b (H-2.33) were prepared as described (Ewenstein et al., 1978; Uehara et al., 1980a).

Isolation of H-2K$^b_{trp}$ (a Fragment Obtained by Limited Trypsin Digestion of the Intact H-2K^b). The glycoprotein fraction purified by lentil lectin affinity column chromatography of NP-40 cell lysates (Brown et al., 1974) was digested by trypsin (trypsin-TPCK, Worthington Biochemical Corp., Freehold, NJ) at 37 °C for 10 min in the presence of bovine serum albumin (usually 0.2 mg/mL) at trypsin concentration of 60-80 μg/mL. The digestion was halted by the addition of an equal amount of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO), and after 30 min at room temperature, H-2K$^b_{trp}$ was isolated by immunoprecipitation (Brown et al., 1974). After reduction and alkylation in 9% $NaDodSO_4$ as described before (Brown et al., 1974), the reaction solution was diluted about 10 times with distilled water and precipitated by the addition of Cl_3CCO_2H at a final concentration of 15%. The Cl_3CCO_2H precipitate was washed successively with 5% Cl_3CCO_2H (2 times), ethanol/ether (1:1 v/v), and ether, after which it was dried at room temperature.

† From the Department of Microbiology and Immunology and the Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461 (H.U. and S.G.N.), and the Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205 (J.E.C.). *Received April 7, 1981.* These studies were supported in part by Grants AI-07289 and AI-10702 from the National Institutes of Health (H.U. and S.G.N.).

[1] Abbreviations used: MHC, major histocompatibility complex; H-2K$^b_{trp}$, H-2K^b glycoprotein derived by trypsin cleavage of the NP-40 solubilized H-2K^b molecule; β_2m, β-2-microglobulin; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin; IgM, immunoglobulin M; V8, *Staphylococcus aureus* V8 protease; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

0006-2960/81/0420-5936$01.25/0

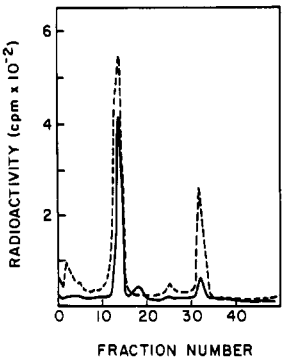

FIGURE 1: Discontinuous polyacrylamide gel electrophoresis in sodium dodecyl sulfate–polyacrylamide gel electrophoresis of [^{3}H]Arg-labeled H-2K$^b_{trp}$ and [^{35}S]Met-labeled H-2K^b. Samples were combined after immunoprecipitation, dissolved in NaDodSO$_4$-containing buffer in the presence of 2-mercaptoethanol, and subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis (Maizel, 1971). (—) ^{3}H; (---) ^{35}S.

Trypsin Digestion and Isolation of Dilute Acid Insoluble Peptides. The Cl_3CCO_2H precipitated H-2K$^b_{trp}$ was suspended in 0.1 M NH_4HCO_3, pH 8.5 (10–15 mg of protein/mL), and TPCK-trypsin was added at a substrate to enzyme ratio of 50:1. After incubation for 1 h at 37 °C, an equal amount of trypsin was added, and the reaction was continued for 3 h. The reaction was halted by adding CH_3COOH to a final 5% of the total volume. Peptides insoluble in 5% CH_3COOH were collected by centrifugation (10000*g* for 10 min) and washed once with 5% CH_3COOH.

Digestion by Staphylococcus aureus V8 protease was performed as described previously (Uehara et al., 1980a).

Gel Filtration of the Dilute Acid Insoluble Peptides. Five percent CH_3COOH insoluble peptides were dissolved in 70% CH_3COOH and applied on a column of Sephadex G-50 (Superfine) (1.5 cm × 140 cm) equilibrated in 50% CH_3COOH; 1.7-mL fractions were collected with a flow rate of 6 mL/h. Chromatography after V8 digestion was performed under identical conditions.

Automated Amino Acid Sequence Analysis. Automated Edman degradation was performed with a Beckman sequencer (Model 890-C) and Beckman Peptide Program No. 102984. The detection of radioactivity, conversion of thiazolinone amino acids to phenylthiohydantoin (PTH) derivatives, and the identification of PTH-amino acids were performed as described previously (Coligan et al., 1979; Uehara et al., 1980a).

Results

Isolation of H-2K$^b_{trp}$. The tryptic fragment, H-2K$^b_{trp}$, which was generated by digestion of the NP-40 cell lysate glycoprotein fraction with trypsin for a short time (trypsin flash) was used as a source for generating the intramembranous segment. H-2K$^b_{trp}$, which was isolated by immunoprecipitation after trypsin digestion, showed a slightly lower molecular weight than the intact H-2K^b molecule whereas β_2m from both preparations is identical in size (Figure 1). Isolation and characterization of the CNBr fragments obtained from H-2K$^b_{trp}$ indicated that H-2K$^b_{trp}$ is a fragment of about 43 000 molecular weight derived by tryptic cleavage at Lys-317 near the COOH terminus of the intact molecule. In addition, as shown in Figure 1, trypsin flash resulted in digestion of high molecular weight material which contaminated the H-2K^b immunoprecipitate. The immunoprecipitate was dissolved in 9% NaDodSO$_4$ and reduced and alkylated, and H-2K$^b_{trp}$ was isolated by precipitation with Cl_3CCO_2H (final 15% w/v).

Isolation of the Acid-Insoluble Tryptic Peptide (TC-1) Containing the Hydrophobic Region. The reduced and alkylated H-2K$^b_{trp}$ was digested by trypsin and the material insoluble in 5% CH_3COOH was isolated by centrifugation, dissolved in 70% acetic acid, and fractionated on a column of Sephadex G-50. As shown in Figure 2A, one distinct Met-containing peak (TC-1:2) was isolated.

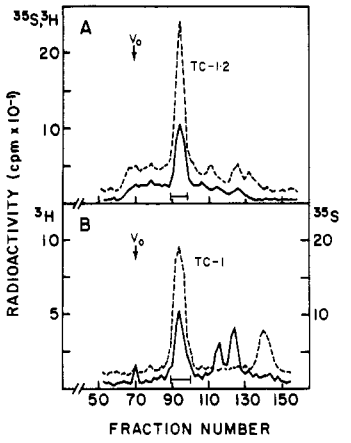

FIGURE 2: Gel filtration of [^{3}H]Thr/[^{35}S]Met-labeled peptides on a column of Sephadex G-50 equilibrated with 50% CH_3COOH. Chromatographic conditions were described under Materials and Methods, V_0 denotes the elution volume of the column. (A) Gel filtration of tryptic peptides insoluble in 5% CH_3COOH. The TC-1:2 pool was made as shown by the bar. (B) Chromatograpohy of V8 protease treated pool TC-1:2. TC-1 was recovered as shown by bar. (—) ^{3}H; (---) ^{35}S.

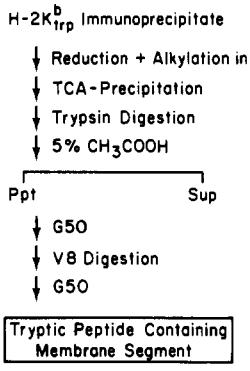

FIGURE 3: Flow chart of the purification scheme of the tryptic peptide TC-1.

Partial sequence analysis of the TC-1:2 indicated that the peak was a mixture of two different peptides (TC-1 and TC-2). It was deduced that TC-1 was the putative intramembranous segment whereas TC-2 was a Cys-containing peptide derived from residues 203–243 of the molecule. Since the size of both peptides is similar, it was difficult to separate these two peptides by gel filtration chromatography. As reported previously (Uehara et al., 1980b), Glu residues were found throughout TC-2 whereas few Glu residues were expected in the intramembranous segment TC-1. Therefore, *Staphylococcus* V8 protease digestion of TC-1:2 was performed in hope that TC-2 would be digested and that TC-1 would remain intact. Chromatography of the V8 digest (Figure 2B) revealed that the peptide TC-1 was not cleaved by the enzyme since it eluted as a pure peptide in the same position as before, whereas TC-2 was cleaved at the Glu residues and the resultant subfragments eluted as several small peptides. (The purification scheme for obtaining TC-1 is summarized in Figure 3).

Sequence Analysis of TC-1. The amino acid sequence of TC-1 is shown in Figure 4. This sequence was obtained from 12 radiolabeled preparations (Figure 5). Each preparation was labeled by [^{35}S]Met and at least one ^{3}H-labeled amino acid. The identification of Met residues at steps 11, 34, and

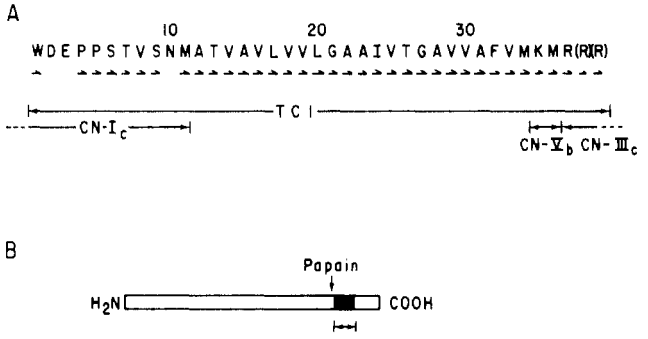

FIGURE 4: (A) Summary of amino acid sequence information for TC-1. Numbers above the sequence refer to residue positions in peptide TC-1. The arrows depict identification of the residues obtained by NH_2-terminal sequence analysis. The positions of CNBr fragments CN-Ic, CN-Vb, and CN-IIIc are also shown. (B) Schematic representation of the location of TC-1 in H-2K^b. The arrow indicates the range of TC-1 and the region shown by black box is the putative membrane binding region.

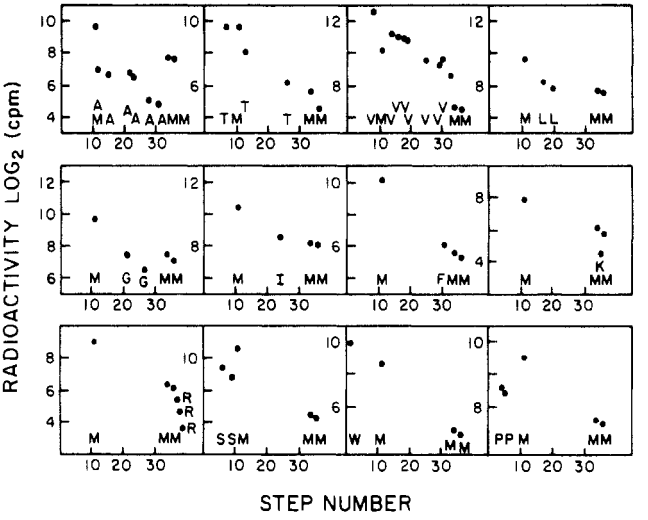

FIGURE 5: Radioactivity above background [as $\log_2$ (cpm)] recovered in each step of the amino acid sequence analysis of TC-1 plotted against step number. The amino acid residue assigned at each position is indicated in the single-letter amino acid code. As shown, 12 radiolabeled preparations were utilized to obtain the sequence of TC-1.

36 was used as an internal control of each sequence determination. As shown in Figure 4A, TC-1 is a peptide composed of 37–39 amino acid residues. The variability in length is due to differences in the number of COOH-terminal Arg residues (see Discussion). Of importance is the finding that TC-1 is the overlapping peptide from the COOH-terminal portion of CNBr fragment Ic (positions 229–284) and the COOH-terminal region of H-2K^b, the sequence of which is described in the accompanying paper (Uehara et al., 1981). The location of TC-1 in H-2K^b is from 274 to 310 (312) (Figure 4B).

Discussion

Sequence analysis of the nine CNBr fragments of H-2K^b isolated as previously described (Ewenstein et al., 1978) revealed that the putative intramembranous segment was not included in these CNBr fragments. Therefore, an alternative procedure for isolating this portion of the H-2K^b molecule was devised.

The tryptic peptide, TC-1, containing the intramembranous segment of H-2K^b was obtained from a tryptic digest of H-2K$^b_{trp}$. Initially, intact H-2K^b molecule purified by gel filtration in the presence of $NaDodSO_4$ was used as the source of TC-1. However, the sequence analysis of TC-1 isolated from the intact H-2K^b molecule was unsatisfactory for an unknown reason(s). Elimination of a Bio-Gel fractionation step in $NaDodSO_4$ was empirically found to provide TC-1 in a form suitable for sequence analysis. Highly purified H-2K$^b_{trp}$ can be isolated without a column chromatographic procedure (see Figure 1). TC-1 was partially purified from a tryptic digest of H-2K$^b_{trp}$ by precipitation with dilute acetic acid (5%). The precipitate was solubilized in 70% acetic acid and fractionated by Sephadex G-50 column chromatography in the presence of 50% acetic acid. Concentrated acid conditions have also been required in structural studies of other membrane-bound proteins such as human glycophorin A (Furthmayr et al., 1978) and cytochrome b_5 (Ozols & Gerard, 1977). TC-1 was contaminated by another hydrophobic tryptic peptide of similar size. However, since TC-1 was resistant to protease V8, it could be isolated in pure form after protease digestion of the contaminanting peptide and subsequent rechromatography.

The tryptic peptide TC-1 is composed of 37–39 amino acid residues and localized from position 274 to position 310 (312) of the H-2K^b molecule. The variability in length is due to the presence of three consecutive Arg residues (positions 310–312 of the molecule) at the COOH terminus of this peptide which trypsin cleaved in variable locations. The mixture of peptides containing 37–39 amino acid residues was eluted at the same fractions in gel filtration (see Figure 2B), and this was analyzed as tryptic peptide TC-1. Within the continuous stretch of 31 uncharged residues found from position 4 to position 34 in this peptide, the region from position 11 to position 34 is noteworthy because of its high hydrophobicity. Within these 24 residues all but the Thr residues at positions 13 and 24 are nonpolar residues. The hydrophobic index (HI) (Segrest & Feldman, 1974) of this region is 2.2, which is the range of the HI (2.56) obtained for the stretch of 23 uncharged residues found in glycophorin A, a major sialoglycoprotein of the human erythrocyte membrane. This hydrophobic stretch in human glycophorin A is postulated to be the membrane-binding region of this transmembrane protein (Bretscher, 1981; Cotmore et al., 1977). A stretch of 23 residues in the α-helix conformation can span a lipid bilayer of approximately 35 Å in thickness (Guidotti, 1977).

Thus, the presence of a highly hydrophobic stretch of 24 residues in TC-1 suggests, although does not prove, that the H-2K^b is a transmembrane protein with residues 285–308 being a membrane-penetrating portion of the molecule. The fact that papain cleavage, which occurs NH_2 terminal to this putative membrane-binding region, releases an antigenically active, water-soluble fragment which no longer reacts with a lipid bilayer is consistent with the loss of the membrane-integrating region (Schwartz & Nathenson, 1971; Ewenstein et al., 1976). The HLA-B7, which is believed to be a transmembrane protein (Walsh & Crumpton, 1978), also contains a stretch of 27 nonpolar residues (Robb, 1978) (see below).

The availability of the partial sequence of the intramembranous segment of the HLA-B7 molecule (Robb, 1978) allows comparison of amino acid sequence of this segment of the H-2K^b and HLA-B7 molecules. As shown in Figure 6, 58% homology was obtained, but only if several gaps were introduced. This value is significantly lower than the 70% homology which was observed for the NH_2-terminal hydrophilic regions of the molecules without introducing any gaps. Although the amino acid sequence of this region of other H-2 molecules has not been determined, the amino acid sequence deduced from DNA sequence of cDNA for a H-2 molecule (pH-2II) (Steinmetz et al., 1981) can be compared with that

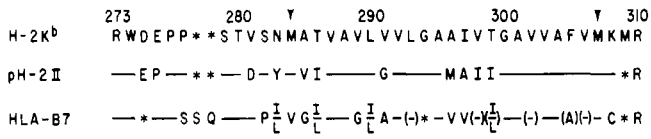

FIGURE 6: Comparison of the amino acid sequence of the membrane-binding regions of H-2K^b, pH-2II, and HLA-B7. The numbers above the H-2K^b sequence refer to positions in the intact H-2K^b molecule. Identical residues are indicated by solid lines and different residues are identified. (*) Gaps required to achieve maximum homologies. Data for HLA-B7 was taken from Robb (1978). The sequence of pH-2II is from Steinmetz et al. (1981).

of H-2K^b (Figure 6): 70% homology was obtained whereas 75–88% homologies were observed among H-2 molecules for the NH_2-terminal 100 residues (Nathenson et al., 1981; Coligan et al., 1981).

The relatively low sequence homology of H-2K^b with HLA-B7 and apparently with other H-2 molecules in the intramembranous region of the molecule may be a reflection of the function of this portion of the molecules. If its sole function is to anchor the molecules into the membrane, it is probably not necessary to retain particular amino acid residues but only hydrophobic residues. Thus, more amino acid sequence differences in this region between two molecules might be accumulated during evolution. In this regard, it is noteworthy that no significant sequence homologies in this region were observed between H-2K^b and glycophorin A (Furthmayr et al., 1978) or membrane-bound IgM (Rogers et al., 1980).

Acknowledgments

We thank Mark Flocco, Diane McGovern, Selma Schmacher, Pat Spinella, Joanne Trojnacki, and Robert Valas for their expert technical assistance and Catherine Whelan for secretarial assistance. We thank our colleagues Ed Kimball, Tom Kindt, Lee Maloy, John Martinko, Roderick Nairn, Larry Pease, and Kazushige Yokoyama for their helpful discussions.

References

Bretscher, M. S. (1981) *Nature (London) New Biol. 231*, 229–232.

Brown, J. L., Kato, K., Silver, J., & Nathenson, S. G. (1974) *Biochemistry 13*, 3174–3178.

Coligan, J. E., Kindt, T. J., Ewenstein, B. M., Uehara, H., Nisizawa, T., & Nathenson, S. G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3390–3394.

Coligan, J. E., Kindt, T. J., Ewenstein, B. M., Uehara, H., Martinko, J. M., & Nathenson, S. G. (1979) *Mol. Immunol. 16*, 3–8.

Coligan, J. E., Kindt, T. J., Uehara, H., Martinko, J. M., & Nathenson, S. G. (1981) *Nature (London) 291*, 35–39.

Cotmore, S. F., Furthmayr, H., & Marchesi, V. T. (1977) *J. Mol. Biol. 113*, 539–553.

Ewenstein, B. M., Freed, J. H., Mole, L. E., & Nathenson, S. G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 915–918.

Ewenstein, B. M., Nisizawa, T., Uehara, H., Nathenson, S. G., Coligan, J. E., & Kindt, T. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2909–2913.

Fleming, P. J., Dailey, H. A., Corcoran, D., & Strittmayer, P. (1978) *J. Biol. Chem. 253*, 5369–5372.

Furthmayr, H., Galardy, R. E., Tomita, M., & Marchesi, V. T. (1978) *Arch. Biochem. Biophys. 185*, 21–29.

Guidotti, G. (1977) *J. Supramol. Struct. 7*, 489–497.

Maizel, J. V. (1971) *Methods Virol. 5*, 179–246.

Martinko, J. M., Uehara, H., Ewenstein, B. M., Kindt, T. J., Coligan, J. E., & Nathenson, S. G. (1980) *Biochemistry 19*, 6188–6193.

Nairn, R., Nathenson, S. G., & Coligan, J. E. (1980) *Eur. J. Immunol. 10*, 495–503.

Nathenson, S. G., Uehara, H., Ewenstein, B. M., Kindt, T. J., & Coligan, J. E. (1981) *Annu. Rev. Biochem. 50*, 1025–1052.

Ozols, J., & Gerard, G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3725–3729.

Robb, R. J. (1978) Ph.D. Thesis, Harvard University, Cambridge, MA.

Rogers, J., Early, P., Cater, C., Calame, K., Bond, M., Hood, L., & Wall, R. (1980) *Cell (Cambridge, Mass.) 20*, 303–312.

Segrest, J. P., & Feldman, R. J. (1974) *J. Mol. Biol. 87*, 853–858.

Snell, G. D., Dausset, J., & Nathenson, S. G. (1976) *Histocompatibility*, Academic Press, New York.

Steinmetz, M., Frelinger, J. G., Fisher, D., Hunkapiller, T., Pereira, D., Weisman, S. M., Uehara, H., Nathenson, S. G., & Hood, L. (1981) *Cell (Cambridge, Mass.) 24*, 125–134.

Takagaki, Y., Gerber, G. E., Nihei, K., & Khorana, H. G. (1980) *J. Biol. Chem. 255*, 1536–1541.

Uehara, H., Ewenstein, B. M., Martinko, J. M., Nathenson, S. G., Coligan, J. E., & Kindt, T. J. (1980a) *Biochemistry 19*, 306–315.

Uehara, H., Ewenstein, B. M., Martinko, J. M., Nathenson, S. G., Kindt T. J., & Coligan, J. E. (1980b) *Biochemistry 19*, 6182–6188.

Uehara, H., Coligan, J. E., & Nathenson, S. G. (1981) *Biochemistry* (following paper in this issue).

Vitetta, E. S., & Capra, J. D. (1979) *Adv. Immunol. 26*, 147–193.

Walsh, F. S., & Crumpton, M. J. (1978) *Nature (London) 269*, 307–311.