Gonzalez, C., Palacios, R., and Schimke, R. T. (1974), *Biochim. Biophys. Acta 366,* 424.
Hamashima, Y., Harter, J. G., and Coons, A. H. (1964), *J. Cell Biol. 20,* 271.
Jamieson, J. C., Morrison, K. E., Molasky, D., and Turchen, B. (1975), *Can. J. Biochem. 53,* 401.
Koj, A. (1970), in Energy Metabolism in Trauma, Porter, R., and Knight, J., Ed., J. and A. Churchill, London, p 79.
Kwan, S.-W., and Webb, T. (1967), *J. Biol. Chem. 242,* 5542.
Murano, G. (1974), *Sem. Thromb. Hemost. 1,* 1.
Palacios, R., Palmiter, R., and Schimke, R. (1972), *J. Biol. Chem. 247,* 2316.
Pawlowski, P. J., Gillette, M. T., Martinell, J., Lukens, L. N., and Furthmayr, H. (1975), *J. Biol. Chem. 250,* 2135.
Reeve, E. B., and Franks, J. J. (1974), *Sem. Thromb. Hemost. 1,* 129.
Sarker, P. K., and Moscona, A. A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1667.
Schecter, I. (1974), *Biochemistry 13,* 1875.
Schubert, D., and Cohn, M. (1968), *J. Mol. Biol. 38,* 273.
Taylor, J. M., and Schimke, R. T. (1974), *J. Biol. Chem. 249,* 3597.
Weimer, H., and Humelbaugh, C. (1967), *Can. J. Physiol. Pharmacol. 45,* 241.

Purification of Murine Thymus Leukemia Antigen (TL). A Quantitative Assessment of Limited Proteolysis†

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ABSTRACT: The murine thymus leukemia antigen (TL) has been solubilized from the tumor ASL1 and from an established cell line ASL1W, by papain digestion. When a 15-min digest was chromatographed on Sephadex G-200, two peaks of TL activity were eluted with apparent molecular weights of approximately 58,000 and 31,000. Chromatography of a 30-min digest under the same conditions resulted in elution of a single peak of activity with an apparent molecular weight of 58,000. Additional purification was carried out on the 58,000 molecular weight material by absorption to, and elution from DEAE-cellulose. The combination of gel filtration and ion exchange chromatography resulted in approximately a 150-fold purification.

Thymus leukemia antigen (TL)[1] has been described serologically by Boyse (Boyse et al., 1968). TL is found normally only on thymocytes of certain mouse strains. However, its expression has been noted on some leukemias of all strains tested, even those that are normally phenotypically TL$^-$. Biochemical studies using radioactive amino acids and sugars have shown TL to be a glycoprotein (Muramatsu et al., 1973). It is apparently made up of two chains. The heavier of the two chains contains the carbohydrate (Muramatsu et al., 1973) and the small chain mol wt 11000–12000 (Ostberg et al., 1975) has been shown to be antigenically cross-reactive with human β_2-microglobulin. These properties are similar to those of mouse transplantation antigens (H-2) (Vitetta et al., 1975; Nathenson and Cullen, 1974; Silver and Hood, 1974).

It has been suggested that TL (Vitetta et al., 1975), along with H-2 (Vitetta et al., 1975) and the human transplantation antigens, HL-A (Strominger et al., 1974), have overall structures similar to immunoglobulins. This suggestion is based on several observations. All of these cell surface antigens have a limited number of sites of papain cleavage, a property which is similar to immunoglobulin. The carbohydrate is found associated only with the heavy chain which is again suggestive of an immunoglobulin-like structure. Sequence analysis of the small chain, β_2-microglobulin, revealed significant homology with immunoglobulins (Cunningham et al., 1973). In addition, HL-A (Strominger et al., 1974; Cresswell, 1975) molecules have been shown to exist as dimers, with the heavy chains joined by disulfide bonds.

Most of the previous studies of TL structure have relied on immunoprecipitation to achieve purification (Muramatsu et al., 1973; Vitetta et al., 1972). Although this allows a quick method of obtaining relatively pure TL, the conditions of release of the TL from the antibody are generally quite severe, e.g., boiling in sodium dodecyl sulfate. This treatment does not leave the molecule in any condition for further biochemical and structural studies. Because of the small amount of TL expressed on cells, there are two principle requirements for further in-depth studies on its structure: (1) a larger source of cells than are available from animals and (2) a method of purification which will leave the TL in a more native state. This report describes the comparative purification of papain-solubilized TL taken from a tumor line (ASL1), and that taken from the established cell line (ASL1W) which has been adapted to spinner culture.

Materials and Methods

Mice. The congenic, A/TL$^-$ strain, was a generous gift from Dr. E. A. Boyse of Sloan Kettering Memorial Institute. Inbred strains, C57Bl/6 (TL$^-$), A/J (TL$^+$), and

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[1] Abbreviations used are: TL, thymus leukemia antigen; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

AKR/J (TL^-), were purchased from Jackson Laboratories, Bar Harbor, Maine. The $(A/TL^- \times C57Bl/6)F_1$ mice were raised in our colony.

Antisera. Alloantisera to TL specificities 1,2,3 were generated by hyperimmunization of $(A/TL^- \times C57Bl/G)F_1$ mice with ASL1 (a gift of Dr. Boyse), a TL 1,2,3 leukemia of A/J mice. The schedule of injections used was that described by Boyse et al. (1968).

Specificity of Antisera. The antisera were tested for cytotoxicity against ASL1 tumor cells, A/J thymocytes and spleen cells, C57Bl/6 thymocytes, and AKR/J thymocytes by the dye exclusion technique (Gorer and O'Gorman, 1956). The antisera were found to be cytotoxic only when tested against A/J thymocytes and ASL1 tumor cells.

Through the use of ^{51}Cr-labeled ASL1 tumor cells as the target, the above cells were tested by absorption for their capacity to specifically inhibit the cytotoxic antisera (Wigzell, 1967; Sanderson, 1964). Only the A/J thymocytes and ASL1 tumor cells were able to absorb the antisera.

Cells. The murine thymus-derived leukemia, ASL1, was first isolated (Old et al., 1963) from an A/J male, and is maintained in vivo by passage of 1.5×10^5 tumor cells intraperitoneally. Death generally occurs at day 10, with each animal yielding approximately 5×10^8 cells. The tumor localizes in the spleen, in the lymph nodes, and in the peritoneal cavity.

The ASL1 tumor line has been adapted to grow in suspension culture in a supplemented Dulbecco's high-glucose modified Eagle's media (GIBCO, Grand Island, New York). The details of growth of this cell line (ASL1W) are reported elsewhere (Williams et al., 1975).

Cytotoxic Assay. Cytotoxic tests were performed by the Trypan Blue exclusion technique (Gorer and O'Gorman, 1956) with the following modifications. Doubling dilutions of 10 μl of antisera were made in microtiter test plates (Falcon), followed by addition of 700–800 cells in 10 μl of Tyrodes, and 10 μl of guinea pig serum as a source of complement. Tests were incubated for 30 min at 37°. At the end of incubation 5 μl of 0.2% Trypan Blue in saline was added. Live and dead cells were counted through the use of an inverted microscope.

Alloantisera were also titered by determining the specific release of ^{51}Cr from labeled ASL1 tumor cells, as described by Wigzell (1967) and Sanderson (1964). Cells were labeled by incubation with $Na_2{}^{51}CrO_4$ (100 μCi/2×10^7 cells) in Tyrodes with 0.1% bovine serum albumin, buffered with 0.02 *M* Hepes (Sigma Chemical Co., St. Louis, Mo.) (pH 7.4). The cells were incubated at 37° for 30 min with gentle agitation, washed twice, and incubated for an additional 30 min at 4° and washed again. After the final wash the cells were resuspended at a concentration of 1×10^7 cells/ml. Doubling dilutions of 20 μl of antisera were made with the Tyrodes solution in microtiter test plates, 10 μl of guinea pig serum was added as a source of complement, and 2×10^5 ^{51}Cr-labeled target cells was added. The final volume in each well was brought to 100 μl with the Tyrodes. Following incubation at 37° for 30 min and centrifugation, a 50-μl aliquot was removed and counted in a γ counter (Searle Analytic). Controls were: cells alone, cells plus antisera, and cells plus complement.

Inhibition of Cytotoxicity. TL activity was followed by inhibition of cytotoxicity. In this procedure a dilution of the cytotoxic antisera was selected which would release 80% of the radioactivity from the labeled target cells. Serial dilutions of intact cells or solubilized TL were made in microtiter plates; 20 μl of the appropriately diluted antiserum was then added to each well and the plates were incubated for 30–45 min to allow absorption of the antibody. Absorption was carried out at room temperature for solubilized TL or 0° for whole cells. After incubation, 20 μl of labeled target cells and 10 μl of guinea pig serum were added and the volume was brought to 100 μl with Tyrodes. The plates were incubated for 30 min at 37°, centrifuged, and 50 μl of supernatant was removed and counted. Inhibition was calculated by:

$$\%I = \left(1 - \frac{\text{cpm specific release with inhibitor}}{\text{cpm specific release without inhibitor}}\right) \times 100$$

One unit of activity is defined as the amount of material which will reduce the antibody mediated release of ^{51}Cr by 50%.

Protein Determination. Proteins were determined fluorometrically after reaction with fluorescamine (Pierce Chem., Rockford, Ill.) according to the method of Bohen et al. (1973). The relative fluorescence of samples was determined on a Fluoro-Microphotometer (Amino, Silver Springs, Md.) equipped with a No. 4-7111 primary filter and a Wratten No. 2 secondary filter.

Proteins were determined relative to a bovine serum albumin standard. The response was found to be lines over a range of 1–7 μg/ml.

Papain Digestion. Papain digestion was carried out as described by Shimada and Nathenson (1967). Cells were suspended at a concentration of 2×10^8 cells/ml in Tris-buffered saline (0.01 *M* Tris–0.14 *M* NaCl (pH 8.0)) and digested with 4 mg/ml of crude papain (Sigma Chemical Co., St. Louis, Mo.) which had been activated with cysteine. Digestion was carried out at 37° with gentle agitation for various periods of time. Following digestion the papain was inactivated by addition of iodoacetamide (in a 1.1 *M* excess with respect to the added cysteine), the digest was centrifuged at 10000 rpm at 4° for 15 min, and the supernatant was removed and centrifuged again at 100000*g* at 4° for 1 hr. The supernatant was then concentrated and dialyzed against phosphate-buffered saline.

Molecular Weight Estimation. Molecular weights were estimated by cochromatography of ^{125}I-labeled (Morrison et al., 1971) protein standards and TL active material on Sephadex G-200 (Andrews, 1964). The elution volumes of the standards were determined by assay in a γ counter and the TL activity was followed by assaying 20 μl of each fraction for inhibition of cytotoxicity. The log molecular weight was plotted vs. elution volume, and the best fit line was calculated by the method of least squares (Lutz, 1973).

Results

Thirty-Minute Digestion of ASL1 Cells. ASL1 cells were harvested from animals and digested for 30 min as described previously in Materials and Methods.

A portion of the digest was initially cochromatographed on Sephadex G-200 with ^{125}I-labeled protein standards (Figure 1); TL eluted from the column as a single peak of activity. Comparison of the elution volume of this TL active material with the radioactive standards gave an apparent molecular weight of 58000. A second portion of the digest was chromatographed exactly in the same manner except that the radioactive standards were omitted. The activity peak from this run was pooled and dialyzed against 0.08 *M* Tris buffer (pH 8.0) which is the starting buffer for DEAE-cellulose chromatography. The TL active material was ap-

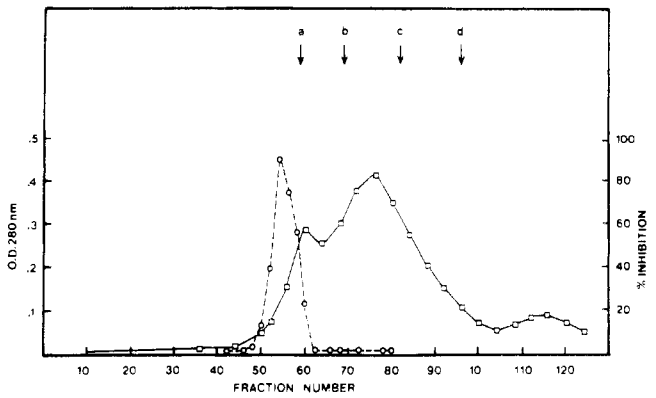

FIGURE 1: Sephadex G-200 column chromatography of the 100000*g* supernatant of the 30-min digest of ASL1. The column was 1.5 × 60 cm eluted with 0.01 *M* phosphate-0.14 *M* NaCl (pH 7.4). The fractions were 1.1 ml. Optical density at 280 nm (—) and TL activity (- - -) was determined for the even numbered fractions. TL activity was assayed by determining the % I of cytotoxicity of a 15-μl aliquot of the fraction. For determination of molecular weight every fraction was counted in a γ counter. The arrows denote the elution point of the radioactive standards: (a) rabbit γ chain, mol wt 50000; (b) pepsin, mol wt 35000; (c) rabbit L-chain, mol wt 22500; (d) ribonuclease, mol wt 13700.

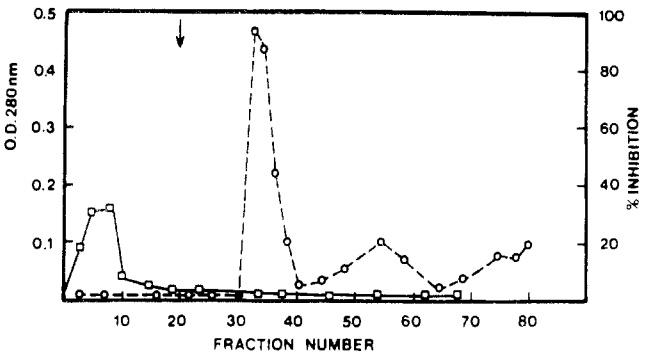

FIGURE 2: Ion exchange chromatography of the TL active fraction from G-200 (Figure 1). The resin was Whatman DE-52 DEAE-cellulose. The active fraction from G-200 was equilibrated with starting buffer (0.08 *M* Tris-HCl (pH 8.0)) and applied to the column (0.9 × 6 cm), and 1-ml fractions were collected. The column was washed with starting buffer until uv absorbing material ceased to be eluted. At fraction 20 (indicated by ↓) a linear gradient was begun from 0.08 *M* Tris-HCl (pH 8.0) to 0.08 *M* Tris-HCl-0.3 *M* NaCl (pH 8.0) and run through fraction 80 (□—□) OD at 280 nm; (○ - - - ○) TL activity.

plied to the ion-exchange column (Figure 2), and the column was washed with starting buffer until there was no detectable uv absorbing material at 280 nm. A linear gradient was begun from 0.08 *M* Tris (pH 8) to 0.08 *M* Tris-0.2 *M* NaCl. The TL eluted shortly after the gradient was begun at a total salt concentration of 0.11 *M*. Portions of the unfractionated digest, the G-200 activity peak, and the active peak from DEAE-cellulose were saved for analysis of specific activity.

Fifteen-Minute Digestion of the Established Cell Line ASL1W. When attempts were made to digest the ASL1W cell line for 30 min with papain, the treatment resulted in almost total lysis of the cells. It was found that 15 min was the longest period of digestion which would leave the cells intact. When the 15-min digest of ASL1W cells was applied to a G-200 column, two peaks of TL activity were noted (Figure 3). When a portion of the digest was cochromatographed on G-200 with radioactive standards the two TL active peaks eluted with apparent molecular weights of 58000 and 31000, respectively. The peak 1 material was applied to DEAE-cellulose (Figure 4) and eluted at the same position in the gradient as the active fraction from the 30-min digest of ASL1. A complex elution profile (Figure 5) was noted when the peak 2 material from Figure 4 was applied to DEAE-cellulose. Although a peak of activity eluted in the same position as that from peak 1 above, activity seemed to be spread throughout the gradient. When this latter material was pooled and concentrated on a P-10 membrane (exclusion 10000 mol wt), no activity was found. Therefore this active material passed through the membrane, i.e., its mol wt was less than 10000 or it was denatured during the concentrating process. In addition, concentrating against a Ficoll gradient through a dialysis bag resulted in no detectable activity.

Fifteen-Minute Digestion of the in Vivo Grown ASL1 Cells. To ascertain whether the second peak of TL activity detected in the papain digest of ASL1W cells was a property of the cell line or was due to the different condition of digestion, a 15-min digestion was carried out on the in vivo grown ASL1. Fractionation of this digest on G-200 (Figure 6) gave two peaks of activity equivalent to the two peaks from the 15-min digest of ASL1W. Further purification of each of these fractions on DEAE-cellulose resulted in an elution pattern similar to the ASL1W material.

Table I: Specific Activities of the Various TL Active Fractions.[a]

Fraction	Specific Activity (units/μg)	Purification (Rel Specific Activity)
30-min digest of ASL1	0.04	1
G-200 peak 1 (Figure 2)	0.44	11
DE-52 peak 1 (Figure 3)	4.13	103
15-min digest of ASL1	0.016	1
G-200 peak 1 (Figure 6)	0.22	14
G-200 peak 2 (Figure 6)	0.067	4
DE-52 peak 1	2.35	147
15-min digest of ASL1W	0.060	1
G-200 peak 1 (Figure 4)	0.77	13
G-200 peak 2 (Figure 4)	0.44	7
DE-52 of peak 1 (Figure 5)	8.90	148

[a] Activities and protein concentrations were calculated as described in Materials and Methods. Relative specific activity is calculated by assigning a value of 1 to the unfractionated digest.

Calculation of Specific Activities. Specific activities (units/μg) were calculated for each of the unfractionated digests, for the G-200 activity peaks, and for the DEAE-cellulose purified 58000 mol wt material. The 31000 mol wt material isolated from DEAE-cellulose was not analyzed for specific activity due to the difficulty in its detection after concentration and dialysis. Following dialysis and concentration to the original volume of the digest, protein concentrations were determined with fluorescamine. Dilutions were made in triplicate of each sample to determine the amount of material which would give 50% inhibition in the cytotoxicity test. The calculated specific activities and degree of relative purification are listed in Table I.

Discussion

The data reported here suggest that TL structure may be evaluated by limited papain cleavage, an approach that has been very successful in studying immunoglobulins. Apparently, while TL remains on the cell, there are at least two sites available for cleavage by papain. Cleavage at one site results in a fragment of approximately 31000 mol wt. This

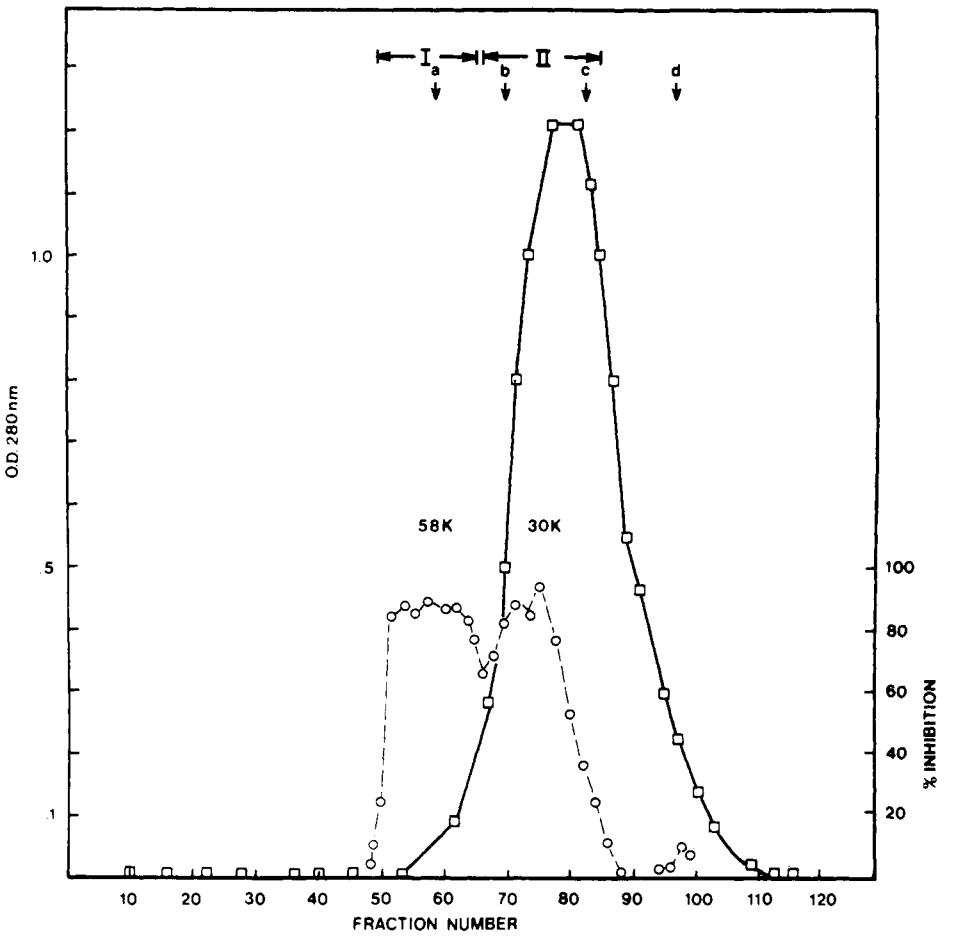

FIGURE 3: G-200 of 100000*g* supernatant 15-min digest of ASL1W. Conditions and legends are same as Figure 1.

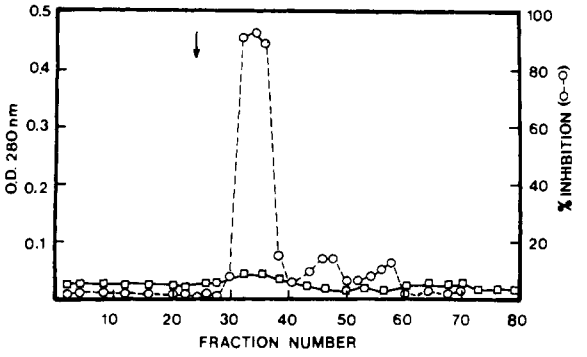

FIGURE 4: DEAE-cellulose chromatograph of peak 1, Figure 3. Conditions and legends are same as Figure 2.

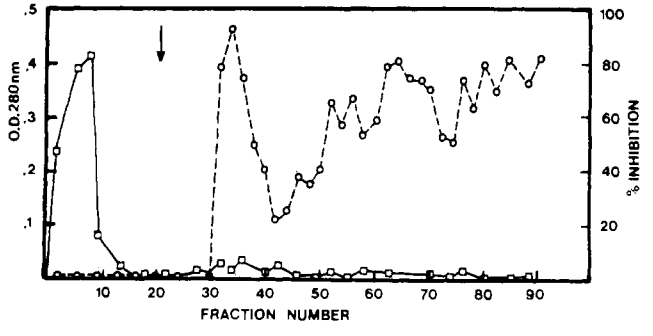

FIGURE 5: DEAE-cellulose chromatography of peak 2, Figure 3. Conditions and legends same as Figure 2.

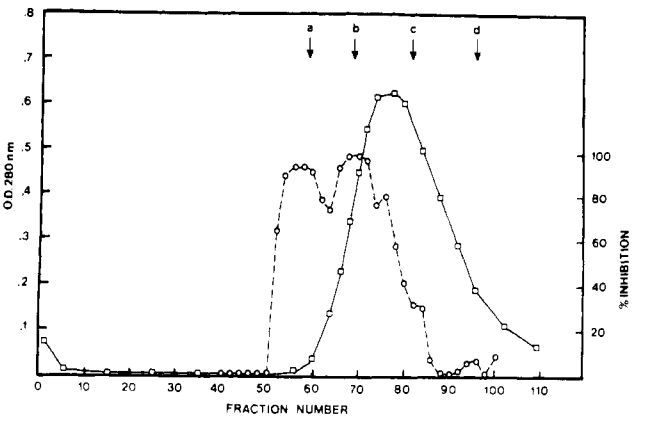

FIGURE 6: G-200 chromatography of the 100000*g* supernatant 15-min digest of ASL1. Conditions and legends are same as in Figure 1.

fragment is not present, or is present only in reduced amounts in the 30-min digest, which suggests that it is prone to continued proteolysis in solution. Cleavage at the second site results in formation of a fragment of approximately 58000 mol wt. This fragment is found in both the 15- and 30-min digests, thus suggesting that it is relatively resistant to continued degradation. One interpretation of these data would be that the cleavage site for production of the 31,000 mol wt fragment becomes cryptic following removal of the larger fragment from the cell surface. A detailed analysis of the kinetics of release of these two fragments and the relative rates of proteolysis of the isolated

fragments by papain is in progress. Data such as these can be gathered only by using material purified in a manner which maintains as much as possible the native configuration of the TL molecules. Previous studies have indicated the association of β_2-microglobulin with the 58,000 mol wt fragment (Vitetta et al., 1975; Ostberg et al., 1975). Although it is unknown whether β_2-microglobulin is associated with the 31,000 mol wt fragment reported here, its absence from this fragment could be a contributing factor to its proteolytic susceptibility. Investigations seeking to clarify this point are in progress.

The methods reported here result in approximately a 150-fold purification. However, no data have been presented on the purity that this represents other than the fact that the DEAE-cellulose purified fraction has been shown to be free of two other cell surface antigens (Thy-1.2 and H-$2K^k$), for which antisera were available (unpublished results). Attempts were made to label the purified TL with ^{125}I, but this procedure resulted in a decrease in specific activity, indicating that some of the TL had been denatured. Therefore, no estimate of purity could be made by determining the percentage of radioactivity which could be precipitated with specific antibody. Currently in progress is work utilizing TL labeled in culture with [^{3}H]leucine to circumvent the problems associated with iodination.

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References

Andrews, P. (1964), *Biochem. J. 91,* 222.

Bohen, P., Stain, S., Dairman, W., and Udenfriend, S. (1973), *Arch. Biochem. Biophys. 155,* 213.

Boyse, E. A., Stockert, E., and Old, L. J. (1968), in International Convocation on Immunology, Rose, N., and Milgrom, F., Ed., Basel, Switzerland, Karger, pp 57–69.

Cresswell, P., and Dawson, J. R. (1975), *J. Immunol. 114,* 523.

Cunningham, B. A., Wang, J. L., Berggard, I., and Peterson, P. A. (1973), *Biochemistry 12,* 4811.

Gorer, P. A., and O'Gorman, P. (1956), *Transplant. Bull. 3,* 142.

Lutz, W. (1973), in Handbook of Experimental Immunology, Vol. 3, Weir, D. M., Ed., Oxford, England, Blackwell Scientific Publications, p A 61.

Morrison, M., Bayse, G. S., and Webster, R. G. (1971), *Immunochemistry 8,* 289–297.

Muramatsu, T., Nathenson, S. G., Boyse, E. A., and Old, L. J. (1973), *J. Exp. Med. 137,* 1256.

Nathenson, S. G., and Cullen, S. E. (1974), *Biochim. Biophys. Acta 344,* 1.

Old, L. J., Boyse, E. A., and Stockert, E. (1963), *J. Nat. Cancer Inst. 31,* 977.

Ostberg, L., Rask, L., Wigzell, H., and Peterson, P. A. (1975), *Nature (London) 253,* 735.

Sanderson, A. R. (1964), *Nature (London) 204,* 250.

Shimada, A., and Nathenson, S. G. (1967), *Biochem. Biophys. Res. Commun. 29,* 828.

Silver, J., and Hood, L. (1974), *Nature (London) 249,* 764.

Strominger, J. L., Cresswell, P., Grey, H., Humphreys, R. E., Mann, D., McCone, J., Parham, P., Robb, R., Sanderson, A. R., Springer, T. A., Terhorse, C., and Turner, M. J. (1974), *Transplant. Rev. 21,* 126.

Vitetta, E., Uhr, J., and Boyse, E. A. (1972), *Cell. Immunol. 4,* 187.

Vitetta, E., Uhr, J., and Boyse, E. A. (1975), *J. Immunol. 114,* 252.

Wigzell, H. (1967), *Transplantation 3,* 423.

Williams, J. L., Stanton, T. H., and Wolcott, M. (1975), *Tissue Antigens* (in press).