The Subunit Structure of Thymus Leukemia Antigens†

Helena Anundi, Lars Rask, Lars Östberg, and Per A. Peterson*

ABSTRACT: EDTA-containing buffer solubilizes thymus leukemia antigens (TLa) from crude thymocyte membrane fractions. The TL antigens consist mainly of molecules of a size similar to immunoglobulin G when gel chromatography analyses were performed under physiological conditions. A single component of TLa was apparent on sucrose density gradient ultracentrifugation of solubilized thymocyte membrane macromolecules as monitored by indirect immunoprecipitation. The sedimentation constant for the TL antigens (5.8 S) was considerably less than that for immunoglobulin G. The gel chromatography and ultracentrifugation data suggest an apparent molecular weight for TLa of about 120000. TLa isolated by indirect immunoprecipitation is composed of two types of polypeptide chains. The smaller subunit was identified as β_2-microglobulin. The larger polypeptide chain carried the alloantigenic determinants and displayed a molecular weight of about 50000 after reduction and alkylation. TLa subjected to molecular weight determination under denaturing conditions was composed of two components. The smaller component was β_2-microglobulin which evidently is linked to the larger polypeptide chain by noncovalent interactions only. The larger component had a size greater than reduced and alkylated immunoglobulin G heavy chains. Upon reduction and alkylation of the latter component its size was reduced and it appeared to have a molecular weight of about 50000. Consequently, TLa is composed of two disulfide linked heavy polypeptide chains and two β_2-microglobulin molecules. TLa solubilized by papain digestion comprises two polypeptide chains, one of which is β_2-microglobulin. The larger 37000-dalton subunit is a fragment of the heavy polypeptide chain. This was demonstrated by digesting solubilized 120000-dalton TLa with papain. The proteolytic fragments obtained were indistinguishable from those directly released from the cell surface by proteolysis. The papain-derived TLa fragment exhibited most if not all the alloantigenic determinants.

In close association to the murine major histocompatibility complex, H-2, a compound locus coding for the leukemia antigens is located (Boyse et al., 1964). These antigens are normally expressed on the surface of thymocytes of some strains of mice. At least three alleles coding for certain antigenic specificities are known for the T-La[1] locus. The antigenic specificities are also sometimes expressed on leukemia cells, also in strains whose thymocytes are TLa negative. The expression of TL antigens on the cell membrane can be modulated in the presence of specific anti-TLa antibodies such that TLa-positive cells may become phenotypically TLa negative. excellent reviews on the expression and genetics of the TL antigens have been published (Boyse et al., 1966; Boyse and Old, 1969).

Despite problems of solubilizing the cell membrane bound TL antigens and the scarcity of material which can be obtained, some information about the molecular characteristics of the TL antigens are available. Davies et al. (1969) were able to show that the TL antigens are similar to H-2 alloantigens. By ion-exchange chromatography they achieved a partial separation of the TL antigens from the H-2 antigens thereby demonstrating that the two antigens are separate molecules. The similarity between the H-2 antigens and the TL antigens was further strengthened in subsequent studies by Muramatsu et al. (1973) who showed that papain-solubilized H-2 and TL antigens were of similar size. The carbohydrate portions displayed, however, distinct differences. Vitetta et al. (1972) and Yu and Cohen (1974) presented evidence that the TL antigens and the H-2 antigens were indistinguishable in molecular weight when solubilized by treatment with nonionic detergent.

Recently, Rask et al. (1974) and Silver and Hood (1974) provided evidence that H-2 antigens are composed of two types of polypeptide chains. The larger subunit carries the alloantigenic determinants, whereas the smaller subunit appears invariant. The latter polypeptide chain seems identical with β_2-microglobulin. In view of the great similarities between H-2 and TL antigens it seemed likely that the TL antigens also might be composed of two types of polypeptide chains. In fact, β_2-microglobulin was shown to be bound also to the TL alloantigenic polypeptide chain (Vitetta et al., 1975; Östberg et al., 1975).

A knowledge of the structure of the TL antigens is of importance for understanding their genetic organization and their possible evolutionary relationship to the H-2 antigens. The present paper describes some characteristics of the TL antigens with particular emphasis on their molecular weight.

Materials and Methods

Source of TL Antigens. Thymocytes from A/Sn mice, carrying the antigenic specificities TL.1,2,3 and from Balb/C mice (TL.2), were obtained from male and female animals of less than 8 weeks of age. For immunosorbent purification of the TL antigens 100–300 mice were sacrificed. In experiments where only analytical work was intended thymus from 10 to 35 animals were used.

Antisera. An antiserum against TL antigens 1 and 3 was raised by injecting (Balb/C × C57Bl/6) F_1 mice (H-2^d, TL.2 × H-2^b, TL−) with A/Sn thymus cells (H-$2K^kD^d$, TL.1,2,3). The antiserum was absorbed with A/Sn spleen

† From the Institute of Medical and Physiological Chemistry, The Biomedical Center, University of Uppsala, Sweden. *Received June 25, 1975.* This work was supported by grants from the Swedish Cancer Society, Marcus Borgströms Fund, and Harald and Greta Jeanssons Fund.

[1] Abbreviations used are: TLa, thymus leukemia antigen; NP-40, Non-Idet P-40; EDTA, ethylenediaminetetraacetic acid; Gdn·HCl, guanidine hydrochloride.

and liver cells and found to be specific for TL antigens after this treatment as described in detail elsewhere (Östberg et al., 1975). Another antiserum reacting with TL.1,3 was prepared by injecting (Balb/C × C_3H/J) F_1 hybrids (H-2^d, TL.2 × H-2^k, TL−) with A/Sn thymocytes (H-$2K^kD^d$, TL.1,2,3). This antiserum reacted exclusively with TL antigens without any absorption. An antiserum against TL.1,2,3 was generously supplied by Dr. E. A. Boyse. Antisera against H-$2K^k$ and H-$2D^d$ alloantigens were prepared by injecting C_3H/J mice (H-2^k) with A/Sn (H-$2K^kD^d$) spleen cells and Balb/C mice (H-2^d) with A/Sn spleen and lymphnode cells. Although the antisera have not been raised in congenic combinations several tests have indicated that they react with TL or H-2 antigens in the expected patterns (cf. Östberg et al., 1975). Antibodies against non-H-2 complex-coded constituents which may be present in the antisera do not seem to be of sufficient affinity to bring down macromolecules by indirect immunoprecipitation. The antiserum against human β_2-microglobulin was the same as earlier described (Östberg et al., 1975). Goat antisera against highly purified rabbit IgG and mouse IgG were raised by subcutaneous injections of the antigens at biweekly intervals for several months. A rabbit anti mouse IgG serum was also prepared by the same immunization schedule.

Solubilization of TL Antigens. Freshly excised thymus was suspended in 0.02 *M* Tris-HCl buffer (pH 8.0). The cells were broken by repeated freezing and thawing. Cell debris, clumps, and remaining intact cells were pelleted at 10000*g* for 10 min. The supernatant fraction was subjected to a second centrifugation at 105000*g* for 60 min. The resulting pellet, henceforth termed crude cell membrane fraction, was taken up either in 0.02 *M* Tris-HCl buffer (pH 7.4) containing 50 m*M* EDTA and 50 m*M* cysteine (for papain digestion) or in 0.02 *M* Tris-HCl buffer (pH 7.4) containing 80 m*M* EDTA, 50 m*M* β-mercaptoethanol, and 2 m*M* phenylmethanesulfonyl fluoride (subsequently called EDTA-containing buffer). The protein concentration was adjusted to 20 mg/ml in both cases.

Papain-solubilization of TL antigens in the crude cell membrane fraction was accomplished by incubating the material with 30 U/ml of crystalline papain (Worthington) for 30 min at 37°C. The incubation was terminated by the addition of iodoacetic acid to a final concentration of 60 m*M*. Particulate material was subsequently removed by centrifugation at 105000*g* for 60 min.

To obtain "intact" TL antigens the thymocyte crude membrane fraction suspended in the EDTA-containing buffer was incubated at +4°C under gentle stirring for 12 hr. Solubilized macromolecules were subsequently obtained by subjecting the incubation mixture to ultracentrifugation at 105000*g* for 60 min.

Radioactive Labeling. Solubilized macromolecules and highly purified TL and H-2 antigens were labeled with ^{125}I or ^{131}I according to the chloramine-T procedure of Hunter and Greenwood (1962).

Assay for TL Antigens, H-2 antigens, and β_2-Microglobulin. The occurrence of TL and H-2 antigens and β_2-microglobulin in solubilized cell membrane molecule preparations and in column effluents was monitored by the indirect immunoprecipitation techniques, as described below. β_2-Microglobulin was also estimated by means of a solid phase radioimmunoassay (Evrin et al., 1971).

The presence of TL antigen-reacting material was also assessed in some cases by taking advantage of the ability of such material to inhibit alloantiserum-induced cytolysis (Gorer and O'Gorman, 1956). The cytotoxicity was scored by the Trypan Blue dye uptake of lysed thymocytes. Experiments were performed at least in duplicate and 200 cells were counted in each analysis.

Isolation of TL Antigens. All TL antigen containing fractions were initially passed over a Sepharose 4B coupled rabbit anti-mouse IgG column. This procedure removed material which adhered nonspecifically to γ-globulin. This step was necessary since subsequent analyses involved immunoprecipitation with various antisera. TL antigens were either recovered directly from the "solubilized" thymocyte macromolecules by indirect immunoprecipitation or by indirect immunoprecipitation following preliminary fractionation of the macromolecules on a column of Sephadex G-200 equilibrated with 0.02 *M* Tris-HCl buffer (pH 8.0) containing 0.15 *M* NaCl. ^{125}I-Labeled solubilized macromolecules were accordingly mixed with antiserum of the desired specificity. The reaction was allowed to proceed for 1 hr at room temperature and for 3 additional hr at +4°C. Immune complexes were precipitated by the subsequent addition of rabbit or goat anti-mouse IgG serum or, when appropriate, goat anti-rabbit IgG serum. The precipitates were collected by centrifugation after overnight incubation at +4°C. The precipitates were washed three times with 0.02 *M* Tris-HCl buffer (pH 8.0) containing 0.1% NP-40 and 1% bovine serum albumin.

For large-scale isolation of a mixture of TL and H-2 antigens the solubilized macromolecules were subjected to gel chromatography on columns of Sephadex G-200 equilibrated with 0.02 *M* Tris-HCl buffer (pH 8.0) containing 0.15 *M* NaCl. The occurrence in the effluent of TL and H-2 antigens was monitored on ^{125}I-labeled aliquots of alternate fractions by indirect immunoprecipitation. Fractions comprising TL antigenic reactivity (and hence H-2 alloantigens, see Results) were pooled and concentrated by ultrafiltration. The TL antigen-containing fraction was subsequently subjected to immunosorbent purification on a Sepharose 4B-coupled anti-β_2-microglobulin antibody column (Cuatrecasas, 1970). Material which passed through the Sepharose column unretarded was subjected to affinity chromatography on a Sepharose-4B coupled human β_2-microglobulin column. Both columns were equilibrated with 0.02 *M* Tris-HCl (pH 8.0) containing 0.15 *M* NaCl. Elution with this buffer was continued until the effluent was devoid of protein. Material retained by the columns was desorbed by applying first a buffer of low pH, 0.02 *M* sodium citrate (pH 3.0) containing 0.5 *M* NaCl, followed by 1 *M* Tris-HCl buffer (pH 8.0). The eluted material was dialyzed against phosphate-buffered saline (pH 7.4) prior to concentration by ultrafiltration.

Molecular Weight Determinations of TL Antigens. Analytical gel chromatography was performed on a Sephadex G-200 column (100 × 1.0 cm) equilibrated with 0.02 *M* Tris-HCl buffer (pH 8.0) containing 0.15 *M* NaCl. The details of this procedure have been outlined elsewhere (Berggård and Peterson, 1969). All experiments were carried out at +4°C, fractions of 1.0 ml were collected and analyses were performed in duplicate. Stokes' molecular radius was calculated from the gel chromatography data as described (Laurent and Killander, 1964). The Stokes' radius of the TL antigens was used in combination with the sedimentation constant to calculate the molecular weight (Siegel and Monty, 1966).

The sedimentation constant for the TL antigens was de-

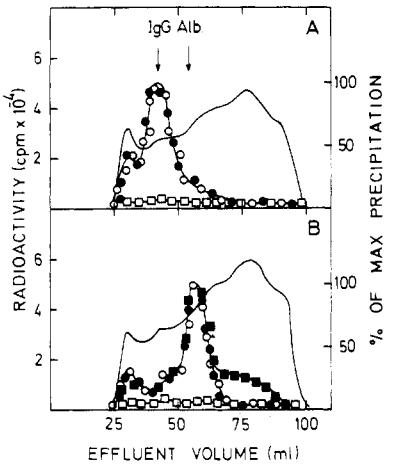

FIGURE 1: Gel chromatography of macromolecules obtained from a crude thymus cell membrane fraction by solubilization with EDTA-containing buffer (A) and by papain digestion (B). Part of a crude cell membrane fraction from A/Sn thymus was solubilized by treatment with the EDTA-containing buffer as described in Materials and Methods, labeled with ^{125}I, and subjected to gel chromatography on a column (108 × 1 cm) of Sephadex G-200 equilibrated with 0.02 *M* Tris-HCl buffer (pH 8.0) containing 0.15 *M* NaCl. Fractions of 1.3 ml were collected at 12-min intervals. Another part of the thymus cell membrane fraction was subjected to proteolytic digestion with papain. ^{125}I-Labeled solubilized macromolecules were subjected to gel chromatography on the same Sephadex G-200 column as described above under identical conditions. The occurrence of TL.1,3 antigens in the effluent was estimated on 0.2-ml aliquots of each fraction by indirect immunoprecipitation. By the same technique the distribution of H-2 alloantigens of H-2D^d specificity and of β_2-microglobulin was monitored. The control precipitates were obtained with normal mouse serum. The arrows indicate the elution positions of immunoglobulin G and albumin which were determined in separate runs. (○) TL antigens; (●) H-2D^d alloantigens; (■) β_2-microglobulin; (□) normal mouse serum; (—) ^{125}I-radioactivity of the solubilized macromolecules.

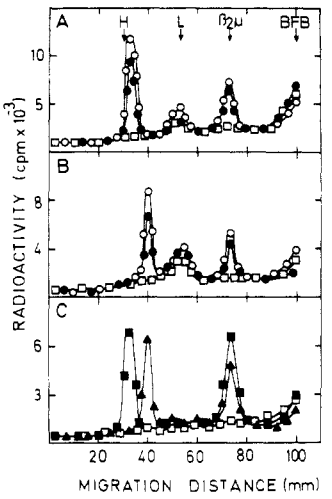

FIGURE 2: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of TL.1,3 antigens and H-2 alloantigens solubilized by treatment with EDTA-containing buffer (A) and papain digestion (B). The materials obtained by indirect immunoprecipitation of solubilized thymus crude membrane macromolecules following gel chromatography on Sephadex G-200 (see Figure 1) were separately reduced and alkylated in sodium dodecyl sulfate and subjected to polyacrylamide gel electrophoresis. In A (○) TL.1,3 antigens, (●) H-2D^d alloantigens, and (□) radioactivity precipitated with normal mouse serum. The same symbols are used in B but the starting material was solubilized by papain treatment. In a separate experiment (C) indirect immunoprecipitation of β_2-microglobulin from solubilized ^{125}I-labeled macromolecules obtained with EDTA-containing buffer (■) and papain treatment (▲) was performed. The anti-β_2-microglobulin serum was substituted for normal rabbit serum to obtain control precipitates (□). The arrows denote the migration positions of marker heavy (H) and light (L) immunoglobulin G chains, β_2-microglobulin ($\beta_2\mu$), and the tracking dye Bromophenol Blue (BFB).

termined by sucrose density gradient ultracentrifugation. The ^{125}I-labeled TL antigen-containing material was applied on to a 5–25% linear sucrose gradient. After 14 hr of centrifugation at 280000*g*, the centrifuge tube was eluted with a micromodification of the device described by Pertoft and Laurent (1969).

The distribution of TL antigens in the gel chromatography and ultracentrifugation experiments was assayed by indirect immunoprecipitation.

Molecular weight determinations of TL antigens under denaturing conditions were performed on sodium dodecyl sulfate polyacrylamide gels (Neville, 1971) or on columns of Sepharose 6B equilibrated with 6 *M* guanidine hydrochloride (Fish et al., 1969). Reduction and alkylation were carried out with 0.01 *M* dithiothreitol and 0.022 *M* iodoacetic acid, respectively.

Other Method. Protein was determined by a modification of the Folin procedure (Lowry et al., 1951). Bovine serum albumin was used as the standard.

Results

Size Distribution of Solubilized TL Antigens. Macromolecules from a thymus crude cell membrane fraction, solubilized by treatment with the EDTA-containing buffer, were labeled with ^{125}I and subjected to gel chromatography on a column of Sephadex G-200 equilibrated with 0.02 *M* Tris-HCl buffer (pH 8.0) containing 0.15 *M* NaCl. The distribution in the effluent of TL alloantigenic and H-2 alloantigenic material was monitored by indirect immunoprecipitation, as shown in Figure 1A. The position of the TL and H-2 antigens was coincident and the main peak of these materials occurred in an elution position corresponding to about that of marker IgG.

A similar experiment was performed with ^{125}I-labeled macromolecules released from thymocyte membranes of papain digestion. It can be seen in Figure 1B that again H-2 and TL alloantigens occurred in similar elution positions after gel chromatography on a column of Sephadex G-200. However, both types of antigens appeared considerably later in the chromatogram than IgG and the elution positions were even slightly later than that for marker albumin. The distribution of β_2-microglobulin was also determined by indirect immunoprecipitation. It occurred in two elution positions the former of which is coincident with that for H-2 and TL antigens.

The above results were obtained with solubilized macromolecules from A/Sn thymocytes carrying antigenic specificities TL.1,2,3. The anti-TLa antiserum employed reacts with antigenic specificities TL.1,3. Therefore, in separate experiments the above procedure was repeated with solubilized thymocyte macromolecules from Balb/C mice (TL.2) and another anti-TLa antiserum reacting with antigenic specificities TL.1,2,3 was used. Results consistent with those shown in Figure 1 were obtained both for macromolecules solubilized with the EDTA-containing buffer and with papain digestion. It seems reasonable to conclude that the antigenic specificities TL.1,2,3 are present on molecules with similar characteristics.

The materials recovered after indirect immunoprecipitation with anti-TLa and anti-H-2 antisera of ^{125}I-labeled macromolecules solubilized by EDTA-containing buffer

Table I: Isolation of TL and H-2 Antigens by Immunosorbent Purification.

Purification Step	Content of TL.1,3[a] Antigens (%)	Content of H-2D^{d}[a] Antigens (%)
Starting material[b]	100	100
Sepharose-anti-β_2-microglobulin		
Unretained	58	62
Bound[c]	14	18
Sepharose-β_2-microglobulin		
Unretained	6	8
Bound[c]	22	16

[a] The content of alloantigens was estimated by their inhibition of alloantisera induced cytolysis. A/Sn thymocytes were used and the viability of the cells was measured by the Trypan Blue dye exclusion. [b] The starting material consisted of 137 thymuses from A/Sn mice of both sexes. [c] This material was desorbed by use of a combination of two buffers (see Materials and Methods). The figures denote the total antigen content in both fractions.

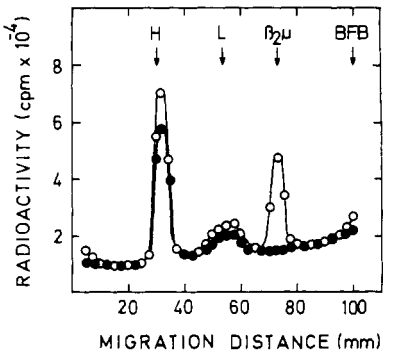

FIGURE 3: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of immunosorbent-purified TL- and H-2 antigen-containing material. Crude thymus membrane macromolecules were solubilized by treatment with the EDTA-containing buffer. The solubilized macromolecules were first subjected to an anti-β_2-microglobulin Sepharose column. Material retained by this column was eluted, labeled with ^{125}I, reduced and alkylated, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Macromolecules which passed the anti-β_2-microglobulin column were subjected to a Sepharose-coupled β_2-microglobulin column. Material bound to this column was desorbed, labeled with ^{131}I, reduced and alkylated, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. (○) Material retained by the anti-β_2-microglobulin column; (●) material bound to the β_2-microglobulin column. The arrows denote the migration positions of extensively reduced and alkylated heavy (H) and light (L) immunoglobulin G chains, β_2-microglobulin ($\beta_{2\mu}$), and the tracking dye Bromophenol Blue (BFB).

and papain digestion, respectively, were separately subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The precipitates were reduced and alkylated in sodium dodecyl sulfate prior to electrophoresis. The distribution of the radioactive material is shown in Figure 2. The precipitated macromolecules obtained after solubilization with the EDTA-containing buffer consisted primarily of one polypeptide chain with an apparent molecular weight of about 50000 whereas macromolecules obtained after papain-digestion displayed a main component in a position corresponding to an apparent molecular weight of about 37000. Both H-2 antigens and TL antigens gave similar electrophoretic patterns (Figure 2). In addition, all immune precipitates contained a small polypeptide chain which migrated identically to marker human β_2-microglobulin. To examine if the small polypeptide chain indeed was the mouse homolog of human β_2-microglobulin, the ^{125}I-labeled macromolecules solubilized by the two procedures were separately subjected to indirect immunoprecipitation with anti-human β_2-microglobulin serum. The reduced and alkylated precipitates were analyzed on sodium dodecyl sulfate polyacrylamide gels. It can be seen in Figure 2C that the size patterns of the two precipitates were very similar to those obtained with anti-TLa and anti-H-2 antigen reagents. Thus, it appears likely that the small polypeptide chain is the mouse homolog to human β_2-microglobulin.

Isolation of TL Antigens by Immunosorbent Purification. A crude cell membrane fraction obtained from A/Sn thymocytes was treated with the EDTA-containing buffer. Solubilized macromolecules nonspecifically sticking to IgG were removed by subjecting the material to filtration on a Sepharose 4B column containing covalently bound rabbit anti-mouse IgG antibodies. Material which passed the column unretarded was subsequently subjected to immunosorbent purification on a column of Sepharose 4B to which rabbit antibodies against human β_2-microglobulin had been covalently bound. Protein adsorbed to the column was eluted with a combination of buffers. The first elution was carried out with 0.02 *M* sodium citrate buffer (pH 3.0) containing 0.5 *M* NaCl which was followed by the second buffer composed of 1.0 *M* Tris-HCl (pH 8.0). The latter buffer had to be employed since some TL antigens precipitate on the column at a low pH but are rendered soluble by the Tris buffer. It can be seen in Table I that about 60% of the TL antigens subjected to the immunosorbent column were not retained. However, only about 15% of the original TL antigen amount could be desorbed from the column by the two buffers as estimated by inhibition of alloantiserum-induced cytotoxicity.

The TL antigens which did not bind to the β_2-microglobulin antibody column were subjected to affinity chromatography on a Sepharose 4B column containing covalently linked human β_2-microglobulin. Only about 6% of the TL antigens passed this column without being bound. The adsorbed protein was eluted with the same combination of buffers as were employed for the antibody column. The eluted material contained about 20% of the original TL antigens. The content of H-2D^{d} alloantigens in the various fractions were also monitored by the inhibition of alloantibody-induced cytolysis. It can be seen in Table I that the recovery of H-2D^{d} antigens was similar to that for TL antigens. The apparent "loss" of TL and H-2 antigens on the affinity chromatography columns most probably is explained by a reduced antigenicity of the molecules due to the drastic elution conditions (see below).

Purity of the Isolated TL Antigens. The materials desorbed from the β_2-microglobulin antibody column and the β_2-microglobulin column were separately labeled with ^{125}I and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis after extensive reduction and alkylation. It can be seen in Figure 3 that the two materials appeared indistinguishable with one notable difference. The small polypeptide chain present in the material recovered from the anti-β_2-microglobulin column did not have a counterpart in the material obtained from the β_2-microglobulin column. The main component in the two materials was, however, a polypeptide chain with an apparent molecular weight of about 50000.

The two labeled materials were subjected to indirect immunoprecipitation with anti-TLa, anti-H-2 D^{d} and K^{k} antigens, and anti-β_2-microglobulin sera. The material recovered from the anti-β_2-microglobulin column was precipitat-

Table II: Molecular Weight Estimations of TL Antigens.

Material[a]	Method	Treatment	Molecular Weight
1. TL.1,3	Sedimentation–diffusion[b]		122000
2. TL.2	Sedimentation–diffusion		116000
3. TL.1,3	SDS[c]		90000; 50000; 12000[e]
4. TL.2	SDS		100000; 50000; 12000[e]
5. TL.1,3	SDS	Reduction + alkylation	50000; 12000
6. TL.2	SDS	Reduction + alkylation	50000; 12000
7. TL.1,2,3 + H-2K^k, H-2D^d	SDS		100000; 50000; 12000[e]
8. TL.1,2,3 + H-2K^k, H-2D^d	SDS	Reduction + alkylation	50000; 12000
9. TL.1,3	SDS	Reduction + alkylation	50000; 12000
10. TL.1,3	6 *M* Gdn·HCl[d]		>80000; >40000; 12000[f]
11. TL.1,3	6 *M* Gdn·HCl		50000[g]
12. TL.1,3	SDS		37000; 12000
13. TL.1,3	SDS	Reduction + alkylation	37000; 12000
14. TL.1,2,3 + H-2K^k + H-2D^d	SDS	Reduction + alkylation	37000; 12000
15. TL.1,2	6 *M* Gdn·HCl	Reduction + alkylation	37000; 12000

[a] The TL antigens were solubilized with the EDTA-containing buffer (1–11) or by papain digestion (12–13). ^{125}I-labeled starting material from A/Sn mice (precipitated with an anti-TL. 1,3 serum) and Balb/C mice (precipitated with an anti-TL. 1,2,3 antiserum) was analyzed (1–6 and 10–13). TL antigens solubilized by treatment with the EDTA-containing buffer were purified by passage over an immunosorbent column containing covalently bound antibodies against β_2-microglobulin, as described in the text (7–9 and 14–15). The isolated material was labeled with ^{125}I and analyzed directly (7 and 8) or after indirect immunoprecipitation with an anti-TL.1,3 antiserum (9). The highly purified material was subjected to papain digestion (14–15) and analyzed directly (14) or after treatment with an anti-TL.1,3 antiserum (15). Further details are given in the text. [b] The sedimentation constant was estimated from analyses in linear sucrose density gradients. The diffusion constant was calculated from analytical gel chromatography data. Details are given in Materials and Methods. [c] SDS denotes sodium dodecyl sulfate polyacrylamide gel electrophoresis. Marker proteins were included in all analyses. [d] Analyses were performed by gel chromatography on columns of Sepharose 6B equilibrated with 6 *M* guanidine hydrochloride. Extensively reduced and alkylated IgG was used as internal marker in each run. [e] The 50000-dalton component occurred in variable amounts in the analyses and its quantity was reciprocal to that for the most high molecular weight molecule. [f] The marker proteins were extensively reduced and alkylated. Accordingly, the molecular weight estimates are minimum values. [g] Analysis performed on the component of molecular weight >80000 obtained from a previous gel chromatography in 6 *M* guanidine hydrochloride (10). Further details are given in the text.

ed by all antisera. However, the alloantisera precipitated only about 15% of the labeled material whereas the antiserum against β_2-microglobulin precipitated about 65% of the labeled material. The reduced and alkylated precipitates were separately subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Two radioactive components were resolved in each case: one small polypeptide chain with size characteristics like those of human β_2-microglobulin and one polypeptide chain with an apparent molecular weight of about 50000.

The precipitates obtained with the ^{125}I-labeled macromolecules obtained from the β_2-microglobulin column contained 12, 16, and 2%, respectively, of the added radioactivity when anti-TLa, anti-H-2 D^d and K^k, and anti-β_2-microglobulin sera were used. When normal mouse serum or normal rabbit serum was used instead of the antisera about 2% of the radioactivity occurred in the precipitates. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the reduced and alkylated anti-TLa and anti-H-2 D^d and K^k sera-induced precipitates revealed in each case a single polypeptide chain with an apparent molecular weight of about 50000.

From these data it appears reasonable to conclude that the two materials recovered from the affinity chromatography columns are homogeneous. Only a minor portion, however, reacts with alloantisera directed against TL antigens and H-2 antigens but such molecules are indistinguishable from the nonantigenic material. This substantiates the results presented in Table I and suggests that the material eluted from the affinity chromatography columns has partially lost the alloantigenic determinants.

Molecular Weight Determinations of TL Antigens. The molecular weight of TL antigens was determined by a combination of gel chromatography and sucrose density gradient ultracentrifugation. A crude cell membrane fraction obtained from A/Sn thymocytes was treated with the EDTA-containing buffer. The solubilized macromolecules were labeled with ^{125}I and subjected to gel chromatography on a column of Sephadex G-200. The occurrence of TL antigens in the eluate was determined on aliquots from each fraction. The TL antigens occurred mainly in an elution position corresponding to that for marker IgG (see Figure 1A). Fractions comprising the TL antigen distribution were pooled and concentrated, and part of the material was subjected to sucrose density gradient ultracentrifugation. After completed sedimentation fractions were assayed for the distribution of TL alloantigenic activity by indirect immunoprecipitation. The TL antigens displayed a single peak but this was somewhat broader than those of marker albumin and IgG. The TL antigen distribution had its maximum distinctly later than that for IgG but ahead of that for albumin. Combining the value for Stokes' molecular radius obtained from the gel chromatography experiment with the sedimentation constant obtained from the sucrose density gradient ultracentrifugation yielded an apparent molecular weight of about 120000 daltons for TL antigens (see Table II).

The ^{125}I-labeled TLa material obtained by indirect immunoprecipitation of the gel chromatography fractions (cf. Figure 1A) was solubilized in 6 *M* guanidine hydrochloride and subjected to gel chromatography on a column of Sepharose 6B equilibrated with the same medium. Figure 4A shows that apart from some aggregated material occurring in the void volume three main radioactive peaks were apparent. The largest component occurred in a position well ahead of reduced and alkylated marker IgG heavy chains. A smaller peak appeared somewhat later than the elution position for the IgG heavy chain and the last eluted radioactive component displayed a size indistinguishable from that of human β_2-microglobulin.

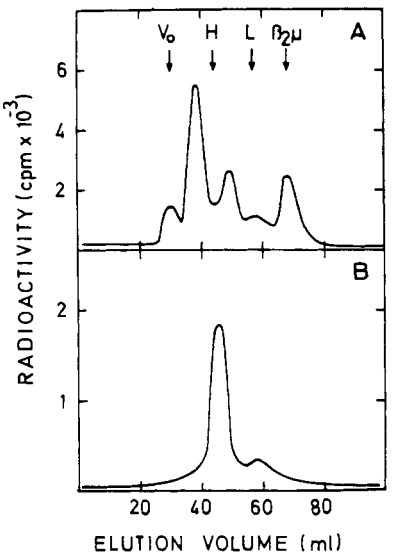

FIGURE 4: Gel chromatography of TL.1,3 antigens in 6 *M* guanidine hydrochloride. TL.1,3 antigens were obtained by indirect immunoprecipitation of ^{125}I-labeled thymus crude membrane macromolecules solubilized by treatment with EDTA-containing buffer as described in the text. The precipitates were dissolved in 6 *M* guanidine hydrochloride and directly subjected to gel chromatography on a column (96 × 1 cm) of Sepharose 6B equilibrated with 6 *M* guanidine hydrochloride (A). The column was operated with a flow rate of 2.2 ml/hr and 1.1-ml fractions were collected. The main radioactive peak depicted in A (occurring in the elution position between 36 and 41 ml) was pooled, concentrated, reduced and alkylated in 6 M guanidine hydrochloride, and rechromatographed on the same column under identical conditions (B). The arrows denote the elution positions of Blue Dextran (V_0), heavy (H) and light (L) immunoglobulin G chains, and β_2-microglobulin ($\beta_{2\mu}$). The marker proteins were completely reduced and alkylated prior to gel chromatography.

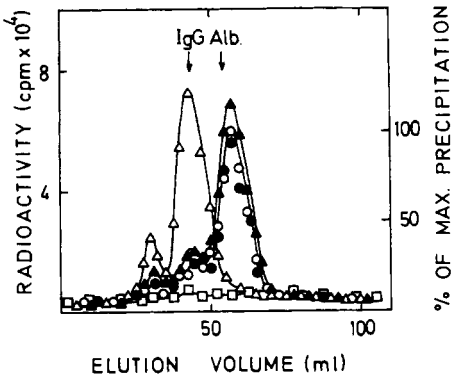

FIGURE 5: Gel chromatography of thymus crude cell membrane macromolecules solubilized by treatment with the EDTA-containing buffer and isolated on an anti-β_2-microglobulin Sepharose column. The material retained by the column was desorbed, labeled with ^{125}I, and part was directly subjected to gel chromatography on a column (106 × 1 cm) of Sephadex G-200 equilibrated with 0.02 *M* Tris-HCl buffer (pH 8.0) containing 0.15 *M* NaCl. The column was operated with a flow rate of 6.0 ml/hr and fractions of 1.4 ml were collected at 14-min intervals. Another part of the ^{125}I-labeled material was digested with 2% papain for 30 min at 37° and subjected to gel chromatography on the same column under identical conditions. Aliquots from each fraction were used to monitor the distribution in the effluent of TL.1,3 and H-2D^d antigens by indirect immunoprecipitation. In control experiments the alloantisera were replaced by normal mouse serum. (△) ^{125}I-labeled macromolecules obtained from the anti-β_2-microglobulin column; (▲) ^{125}I-labeled macromolecules obtained from the anti-β_2-microglobulin column after papain digestion; (○) distribution of TL.1,3 antigens; and (●) H-2D^d antigens among the papain-digested macromolecules; (□) radioactivity in control precipitates obtained with normal mouse serum. The arrows denote the elution positions of immunoglobulin G (IgG) and albumin (Alb.), determined in separate runs.

The main peak occurring earlier than the IgG heavy chains was pooled, concentrated, reduced, alkylated, and resubjected to chromatography on the same column. As can be seen in Figure 4B, the radioactivity now occurred later in the chromatogram and the single peak appeared in about the same position as the marker heavy chains. This result indicates that most of the TL antigens occur as disulfide-linked dimers.

All molecular weight estimations are summarized in Table II. It can be seen that the material recovered from the anti-β_2-microglobulin immunosorbent column exhibited molecular weight characteristics similar to those noted for the immunoprecipitated TL antigens. Since the immunosorbent-purified material appears to contain mainly TL and H-2 alloantigens, it seems reasonable to conclude that the TL antigens and the H-2 antigens display very similar size and weight characteristics.

Relationship between TL Antigens Solubilized by EDTA-Containing Buffer and Papain Digestion. It can be seen in Figure 1 that TL antigens solubilized by papain digestion are of a size considerably smaller than that of the TL antigens solubilized by treatment with the EDTA-containing buffer. To examine the relationship between the two types of molecules material recovered from the anti-β_2-microglobulin column was labeled with ^{125}I and digested with papain. Analysis of the material on Sephadex G-200 gel chromatography after proteolysis revealed that most of the radioactivity appeared in an elution position somewhat later than that for marker albumin (Figure 5). It can also be seen in the figure that the new peak generated by the papain digestion contained molecules with TL and H-2 alloantigenic determinants, as monitored by indirect immunoprecipitation. The precipitates were dissolved in 6 *M* guanidine hydrochloride and part of the material was directly subjected to gel chromatography under denaturing conditions whereas the rest of the precipitated material was reduced and alkylated prior to gel chromatography. Figure 6 demonstrates that the untreated papain-digested material displayed two peaks occurring in elution positions corresponding to approximate molecular weights of about 30000 and 10000, respectively. The employed method gives accurate molecular weight information only if the marker proteins and the examined components display the same conformation. For proteins this may generally be achieved by their assuming random coil conformation in 6 *M* guanidine hydrochloride. A prerequisite for this conformational state, however, is that all covalent interactions but the peptide bonds are disrupted. Accordingly, a protein with intact intrachain disulfide bonds would have a more compact structure than when in random coil conformation. That the two TL antigen polypeptide chains contain intrachain disulfide bridge(s) was apparent since after reduction and alkylation (Figure 6B) the molecular weights of the radioactive polypeptide chains increased to about 37000 and 12000, respectively. In a separate experiment, reduced and alkylated ^{125}I-labeled TL antigens were mixed with ^{131}I-labeled, untreated TL antigens and subjected to chromatography in 6 *M* guanidine hydrochloride. The elution pattern confirmed that the reduced and alkylated polypeptide chains indeed emerged in earlier elution positions than their untreated counterparts.

From this experiment it is apparent that the molecular weight of the proteolytic fragments generated by papain digestion was about 50000, assuming that the two polypeptide

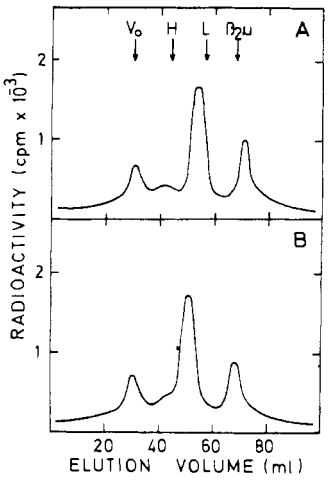

FIGURE 6: Gel chromatography of TL.1,3 antigens in 6 *M* guanidine hydrochloride. ^{125}I-Labeled TL.1,3 antigens were obtained by indirect immunoprecipitation of papain-digested macromolecules which had been solubilized by treatment with the EDTA-containing buffer and isolated on an anti-β_2-microglobulin Sepharose column (see Figure 5). Part of the precipitated material was dissolved in 6 *M* guanidine hydrochloride and directly subjected to gel chromatography on a column (93 × 1 cm) of Sepharose 6B equilibrated with 6 *M* guanidine hydrochloride (A). Another part of the precipitated TL.1,3 antigens was extensively reduced and alkylated in 6 *M* guanidine hydrochloride prior to separation on the same Sepharose 6B column (B). The flow rate of the column was 2.4 ml/hr and fractions of 0.8 ml were collected at 20-min intervals. The arrows denote the elution positions of Blue dextran (V_0), heavy (H) and light (L) chains of immunoglobulin G, and β_2-microglobulin ($\beta_2\mu$). The marker proteins were reduced and alkylated in 6 *M* guanidine hydrochloride prior to chromatography.

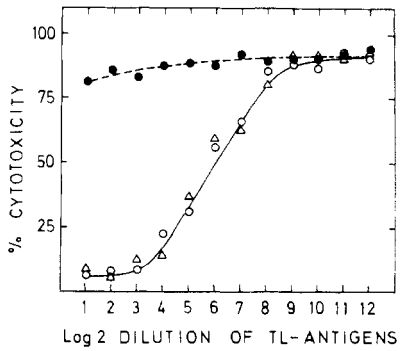

FIGURE 7: Inhibition of cytotoxicity of an anti-TL.1,3 antiserum. Material obtained from thymus crude membranes solubilized by EDTA-containing buffer was isolated on an anti-β_2-microglobulin Sepharose column. Part of the material was digested with papain and the cleavage products were purified by gel chromatography on a column of Sephadex G-200. The material recovered from the anti-β_2-microglobulin column (○) and its papain-digested product (△) were adjusted to similar concentrations and serial dilutions of the two materials were separately examined for inhibition of TL.1,3 antiserum-induced cytolysis. To 25 μl of antigenic material was added 50 μl of an antiserum dilution which gave 90% cytotoxicity on A/Sn thymus cells. After 30 min at +4°C, 50 μl of cells (2×10^6 per ml) and 50 μl of 1/10-diluted agarose-absorbed rabbit serum were added. The incubation was continued for another 30 min at +37°C. After this time period the tubes were placed on ice and 50 μl of 0.2% Trypan Blue dye was added. At least 200 cells were scored for dye uptake in each sample and all dilutions were tested in duplicate. As a control (●) spleen cell macromolecules from A/Sn mice eluted from an anti-β_2-microglobulin column were used.

chains occurred in an equimolar ratio. Furthermore, it is obvious that the two components are not held together by interchain disulfide bonds since they separated even in the absence of reduction and alkylation.

The proteolytic fragments derived from papain digestion of the ^{125}I-labeled material obtained from the anti-β_2-microglobulin column were mixed with ^{131}I-labeled macromolecules solubilized by papain treatment of a crude cell membrane fraction from A/Sn thymocytes. The mixture was subjected to chromatography on a column of Sephadex G-200 and the distribution in the effluent of TLa-reacting material was assayed by indirect immunoprecipitation. A single peak of ^{131}I radioactivity, coincident with that of the ^{125}I-labeled isotope, was precipitated by the antiserum. Reduced and alkylated precipitate was analyzed on sodium dodecyl sulfate polyacrylamide gel electrophoresis. Both the ^{125}I and the ^{131}I radioactivity gave rise to two peaks, one corresponding in size to human β_2-microglobulin, the other with an apparent molecular weight of about 38000.

TLa fragments derived from papain digestion of the anti-β_2-microglobulin column purified material (see Figure 6) were compared with the intact material with regard to alloantigenicity. Both materials were used to inhibit the anti-TLa serum induced cytotoxicity. It can be seen in Figure 7 that both materials were able to completely inhibit the cytotoxic action of the antiserum in a similar fashion. It seems reasonable to conclude that most if not all of the alloantigenic determinants are retained on the fragment derived by papain digestion.

Discussion

The TL antigens are cell surface-bound molecules which poses certain problems for their isolation and characterization. Since these molecules most probably are integrated into the hydrophobic matrix of the cell membrane difficulties arise in rendering the TL antigens water soluble. Several investigators have shown that this can be accomplished by papain digestion of the cell membrane-bound macromolecules (Davies et al., 1969; Muramatsu et al. (1973). It is, however, to be expected that only fragments of the intact TLa molecule will be released by such a solubilization procedure, as shown in the present study. Other workers have employed nonionic detergents to release the membrane-bound TL antigens (Vitetta et al., 1972; Yu and Cohen, 1974; Vitetta et al., 1975; Östberg et al., 1975). This procedure is effective and the solubilized antigens are larger than the papain-solubilized TL antigens. Several difficulties are apparent, however, with NP-40 solubilized macromolecules. The antigens bind a considerable amount of the detergent which renders analyses under physiological conditions hard to interpret. In our hands such material also displays a great tendency to aggregate. Furthermore, the NP-40 treatment seems to release considerable proteolytic activity and the TL antigens are degraded to a great extent already after a few days at +4°C. The method employed to solubilize TL antigens in the present study provides some advantages. No detergent is employed and the solubilized macromolecules are aggregated to a lesser extent. However, the effectivity of solubilization is far less than that obtained with NP-40. Probably only about one-tenth the amount of TL antigens are solubilized as compared to NP-40 treatment.[2] For the present purpose it seemed advantageous to obtain TL antigens with suitable physical-chemical properties rather than to maximize the yield of antigen.

Mainly two methods have previously been used in attempts to purify TL antigens. Davies et al. (1969) and Muramatsu et al. (1973) separated TL antigens on gel chromatography and ion exchange chromatography. Although the

[2] K. Sege et al., in preparation.

TL antigens appeared relatively homogeneous with regard to size they displayed great charge heterogeneity (Davies et al., 1969). A more fruitful approach is provided by the indirect immunoprecipitation of TL antigens (Vitetta et al., 1972; Yu and Cohen, 1974). This method, however, is limited by the tedious procedure to obtain large amounts of specific antisera and the precipitated antigens have to be dissolved under drastic conditions. In addition to indirect immunoprecipitation, we have therefore used immunosorbent purification. Since the TL antigens contain β_2-microglobulin (Vitetta et al., 1975; Östberg et al., 1975) an anti-β_2-microglobulin antibody column was used. Although antibodies against mouse β_2-microglobulin were not available, some antisera against the human protein cross-reacts with the mouse homolog well enough to be employed (Rask et al., 1974). The anti-β_2-microglobulin antibody column did not retain all TL antigens. This result most probably depends on the previously reported finding that some anti-β_2-microglobulin antibodies dissociate β_2-microglobulin from the alloantigenic polypeptide chain (Östberg et al., 1974; Lindblom et al., 1974). Support for this notion was obtained by the observation that TL antigens which passed the anti-β_2-microglobulin antibody column did bind to a β_2-microglobulin column. It is therefore likely that most of the β_2-microglobulin was removed from the TL antigens and retained on the anti-β_2-microglobulin column since only negligible amounts of TL antigens will bind to the β_2-microglobulin column if the material has not been passed over the anti-β_2-microglobulin first.[2]

The material recovered from the affinity chromatography columns was indistinguishable from TL and H-2 antigens precipitated with specific alloantisera. The antigenicity of the material was, however, greatly reduced. This is most easily explained by the drastic elution conditions employed. Current efforts are aimed at finding more specific and less deranging solvents for the elution of bound alloantigens. In spite of the poor antigenicity enough antigenic determinants were present on the immunosorbent-purified molecules to allow a positive identification of TL and H-2 antigens in the material.

One aim of the present study was to define the molecular weight of TL antigens. Unfortunately, available technology is inadequate to obtain enough of highly purified, "native" TL antigens which would be suitable for high precision molecular weight determinations in the analytical ultracentrifuge by sedimentation-equilibrium methods. Since isolation procedures of TL antigens mostly involve precipitation with specific antibodies, the immune complexes have to be dissolved, preferably under denaturing conditions, before analyses of the molecular weight can be performed. Alternatively, molecular weights may be estimated by a combination of methods, such as analytical gel chromatography and sucrose density gradient ultracentrifugation, which allow the identification of the TL antigens present in a crude mixture. The latter methods as well as sodium dodecyl sulfate polyacrylamide gel electrophoresis and gel chromatography in 6 *M* guanidine hydrochloride, which have been used in the present study, although highly reproducible are not comparable in precision to, e.g., sedimentation-equilibrium ultracentrifugation. They are, however, well-founded techniques and, since they are dependent on different properties of the examined protein, a combination of these methods should yield molecular weight estimates that are reasonably accurate.

The TL antigens solubilized with the EDTA-containing buffer are of a size similar to IgG and H-2 alloantigens. They consist of two types of polypeptide chains one of which is β_2-microglobulin, as evidenced by indirect immunoprecipitation. This is in agreement with previous findings (Vitetta et al., 1975; Östberg et al., 1975). The larger polypeptide chain which carries the specific TL antigenic determinants is obviously indistinguishable in molecular weight from the alloantigenic H-2 polypeptide chain.

The size of TL antigens as determined by gel chromatography and their behavior on sucrose density gradient ultracentrifugation suggest an apparent molecular weight of about 120000. On the assumption that β_2-microglobulin forms a complex with the alloantigenic polypeptide chain exhibiting 1:1 stoichiometry, it appears reasonable that the TL antigen molecule is composed of four polypeptide chains, two of each type. This appears all the more likely in view of the molecular weight estimates presented here. The alloantigenic polypeptide chain has an apparent molecular weight of about 50000 under denaturing conditions of both on electrophoretic and gel chromatographic examination. These data are consistent with several previous investigations (Vitetta et al., 1972, 1975; Yu and Cohen, 1974; Östberg et al., 1975). A four-polypeptide chain structure for the TL antigens would accordingly yield an approximate molecular weight of 124000 which is in good agreement with the present observations. A subunit structure for TL antigens comprising, e.g., four molecules of β_2-microglobulin paired with the two heavy chains would yield an approximate molecular weight of 150000. Such a structure cannot be refuted on basis of the sedimentation-diffusion molecular weight data only considering the precision of the methods employed. However, papain digestion of EDTA-solubilized TL antigens yielded fragments composed of one molecule each of β_2-microglobulin and the heavy chain fragment (see below). Since no free β_2-microglobulin was generated by this treatment and since β_2-microglobulin is very resistant toward proteolysis, these data strongly argue for a four-polypeptide chain structure. That two heavy alloantigenic polypeptide chains comprise the predominant part of the TL antigens was clearly shown since a substantial portion of these chains occurred as disulfide linked dimers. A minor fraction was, however, present as monomers under denaturing conditions even in the absence of reducing agents. Whether this material represents a subpopulation of the TL antigens or merely reflects the lability of the disulfide bond(s) between the heavy chains is impossible to ascertain from the present data. Alternatively, the disulfide bond(s) may have been generated by the solubilization conditions. Previous findings with regard to H-2 and HL-A antigens have also revealed dimeric as well as monomeric alloantigenic polypeptide chains (Schwartz et al., 1973; Cresswell and Dawson, 1975; Peterson et al., 1975).

The TL antigens solubilized by papain digestion were of a size considerably smaller than the TL antigens solubilized by the EDTA-containing buffer. The proteolytically derived fragments were composed of two polypeptide chains. The smaller one was identified as β_2-microglobulin and consequently the larger chain represented about 75% of the alloantigenic polypeptide chain. Digestion with papain of the highly purified TL antigens solubilized by treatment with the EDTA buffer yielded TLa fragments with size and weight characteristics similar to those of TL antigens solubilized from the cell surface by proteolysis. It is not possible to distinguish between the two possible modes of action of the papain in solubilizing the macromolecules. Thus, papain

may first release the TL antigens by degradation of vicinal structures on the cell surface and subsequently remove part of the solubilized TL antigens or papain may act directly on the TL antigens leaving a portion of the molecule in situ. It is evident, however, that papain preferentially digests the portion of solubilized TL antigens which contains the interchain disulfide bridge(s). Circumstantial evidence suggests that it is this part of the TL antigens which is situated in the cell membrane. This may be inferred from the fact that the TLa papain fragments display all or most of the alloantigenic determinants. Furthermore, removal of the disulfide bond containing portion obviously renders the TL antigens more water soluble.

The TL antigens are coded for by a locus located adjacent to the H-2 complex. The present information reveals that H-2 alloantigens and TL antigens have similar molecular characteristics. Both are tetrameric molecules (Peterson et al., 1975) and contain β_2-microglobulin as one of the two types of subunits. Although the H-2 and TL alloantigenic polypeptide chains are immunologically distinct they display similar molecular weights. Furthermore, both polypeptide chains are digested by papain similarly and, thus, yield proteolytic fragments of indistinguishable size. It is commonly believed that the H-2 complex arose by events of gene duplications (see Klein and Shreffler, 1971). The present observations would suggest that also the TLa locus may have arisen by the same process. Recent observations have shown that H-2 antigens share several features with immunoglobulins (Peterson et al., 1975) and it has been postulated that H-2 antigens and immunoglobulins are evolutionarily related (Gally and Edelman, 1972; Peterson et al., 1975). It now appears possible that also the TL antigens may share this similarity with immunoglobulins.

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