SOME BIOCHEMICAL PROPERTIES OF THYMUS LEUKEMIA ANTIGENS SOLUBILIZED FROM CELL MEMBRANES BY PAPAIN DIGESTION*

By TAKASHI MURAMATSU,‡ STANLEY G. NATHENSON,§ EDWARD A. BOYSE, and LLOYD J. OLD

(*From the Departments of Microbiology and Immunology and Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461, and Sloan-Kettering Institute for Cancer Research, New York 10021*)

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The thymus leukemia (TL)[1] alloantigens are expressed on the cell surfaces of murine thymocytes and on certain murine leukemia cells. They are called differentiation antigens since in the normal state they are expressed on one cell type, the thymocyte. In addition, some TL specificities are detected on leukemia cells, but not on the thymocytes of the mouse strain from which the leukemia is derived, and hence the antigens also belong to the group of antigens called tumor specific (1).

A noteworthy feature of the *Tla* locus controlling the expression of the TL antigens is its close linkage to the *H-2* genes. Also, there appears to be a cell surface topographical relationship of the TL products to the products of the *H-2D* locus as shown by antibody-blocking techniques (2). Another interesting feature of the *Tla* system is the phenomenon of antibody-mediated antigen disappearance, a process termed antigenic modulation (1).

Although earlier studies have suggested the possible glycoprotein nature of the TL antigens, and certain similarities to the H-2 gene products (3), the TL antigens have not been extensively purified and studied for their chemical characteristics. Using techniques that were developed for studies on the biochemical properties of H-2 alloantigens, we have now solubilized the TL alloantigen from the membrane by proteolytic cleavage with papain (4) and isolated it by indirect immunoprecipitation (5). We present evidence herein that the TL antigen so isolated is a glycoprotein fragment with a mol wt similar to the H-2 glycoprotein fragments isolated by the same procedures; however, the glycopeptide isolated from the TL antigen is of a mol wt different from that of the H-2 glycopeptide.

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[1] *Abbreviations used in this paper:* SDS, sodium dodecyl sulfate; TL, thymus leukemia.

Materials and Methods

Tumor Cells.—RADA1 cells (TL.1, 2, 3) were used as the source of TL antigen. RADA1 is a radiation-induced leukemia adapted to the ascites form and passaged in strain A mice.

Labeling of Cells.—RADA1 cells were incubated at 2×10^6/ml in 500 ml of Joklik modified essential medium (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal calf serum. Labeling with [^{3}H]fucose (500 μCi) or with [^{14}C]amino acid mixture (100 μCi) was identical except that for the latter studies the medium was prepared with $\frac{1}{20}$th the level of the cold amino acids. After 15 h incubation the cells were processed.

Papain Digestion.—Radiolabeled RADA1 cells (approx. 2×10^6/ml in 500 ml of medium) were collected by centrifugation and suspended in 4 ml of Hanks' balanced salt solution. 20 mg of crude papain (Sigma Chemical Co., St. Louis, Mo.) in 1 ml of 0.3 M Tris-HCl buffer, pH 8.4, containing 0.05 M cystine was added to the cell suspension. The reaction was continued at 37°C for 30 min with occasional shaking and was stopped by the addition of 0.05 ml of 2.0 M sodium monoiodoacetic acid. After centrifugation at 10,000 rpm for 1 h the supernatant was removed. This supernatant was called the papain-solubilized extract from the cells. Glycopeptides were prepared as described previously (6) using pronase for digestion.

Antisera.—The antiserum used for these studies to detect TL.1, 2, 3 was A/TL$^-$ (congenic strain) anti-A strain TL$^+$ leukemia ASL1. The antisera to detect anti-*H-2* activity were raised in (AKR $\times$ C57BL/6)F_1 hybrids (*H-2*^{k} $\times$ *H-2*^{b}) injected with Meth-A (a chemically induced fibrosarcoma of BALB/c mice, *H-2*^{d}), or were raised in strain A mice (*H-2*^{a}) injected with Meth-A. The first H-2 serum, when measured on A strain cells, will detect antigen H-2.4 primarily, which is a determinant found on the product of the *H-2D* gene of the *H-2*^{a} haplotype; the second H-2 serum will detect H-2.31, which is not found on cells of the *H-2*^{a} haplotype.

Assay Systems.—The cytotoxic assay for TL activity was carried out using preadsorbed guinea pig complement in RPMI 1640 medium (Grand Island Biological Co.). The cytotoxic test for H-2 activity was carried out as described (7). The indirect precipitation assay for TL activity or for H-2 activity was carried out as previously described, except that papain-solubilized antigen rather than Nonidet P-40-solubilized radiolabeled antigen (Nonidet P-40, Shell Chemical Co., Ltd., London, England) was used (5).

For assay of the columns, 0.3 ml of each fraction was mixed with 0.01 ml of anti-TL antiserum (A/TL-anti-ASL1) or 0.01 ml of a control hyperimmune serum (A anti-Meth-A). The resulting complexes were recovered by precipitation with 0.1 ml of a goat antimouse IgG serum and washed and counted as described (5).

Other Methods.—Protein determination was carried out by the method of Lowry et al. (8), and all chemicals were reagent grade or better. Radioactivity was determined using 5 ml of Aquasol (New England Nuclear, Boston, Mass.) in mini-vials counted with an adapter on a Beckman LS 250 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

RESULTS AND DISCUSSION

Papain proteolytic digestion of intact cells, a procedure previously shown to release the H-2 alloantigens from their membrane site (4), was also successful in releasing material reactive in the cytotoxic inhibition test for TL.1, 2, 3. This preparation could then be fractionated by Sephadex G-150 molecular sieve column chromatography as shown in Fig. 1. When assayed by the inhibition of immune cytolysis of ^{51}Cr-labeled thymocytes by anti-TL antibody, TL antigenic activity was detected with a peak about tube 28 and 29 between the excluded and included volume of the column. When the [^{3}H]fucose-labeled eluate fractions were assayed by the indirect precipitation method (5) in which ali-

quots of the column fractions were mixed with anti-TL antiserum and the antigen-antibody complex was precipitated by goat antimouse gamma globulin serum, a peak was found that clearly coincided with the TL antigenic peak measured by the cytotoxic assay (Fig. 1). Thus, we can conclude that [^{3}H]fucose is incorporated as a part of the TL antigen since there is coincidence of the pattern of activity measured by the cytotoxic inhibition assay and by the indirect precipitation assay. Further support for this conclusion is the expected finding that for the peak tubes, the radioactivity of the specific precipitate (using the anti-TL serum) was 8–10 times that of the nonspecific precipitate (using an anti-H-2.31 serum that is not positive with A/J cells).

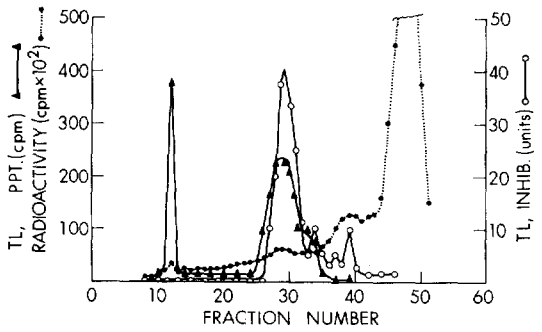

FIG. 1. Sephadex G-150 column chromatography of crude papain-solubilized TL antigen. RADA1 cells were radiolabeled in suspension culture with [^{3}H]fucose as described in the section on Materials and Methods, and then were collected and subjected to papain digestion. The papain-solubilized supernatant was applied to a column of Sephadex G-150 (1.5 × 58 cm) that was equilibrated and eluted with 0.01 M Tris-HCl buffer, pH 8.5, containing 0.15 M NaCl. 2.0-ml samples were collected and analyzed as follows: (*a*) cytotoxic assay of TL activity (○—○) measured by inhibition of release of ^{51}Cr from labeled thymocytes of strain A mice by an anti-TL serum (A/TL$^-$ anti-ASL1); (*b*) indirect precipitation assay of TL activity (▲—▲) and total radioactivity (● · · · ●). Both assays detected TL in coincident peaks. The peak at tube 12 noted in the indirect precipitation assay was nonspecific since the ratio of radioactivity in specific and nonspecific precipitates was about 1.3.

H-2 alloantigens are solubilized by the papain treatment, and for the fractions of the column shown in Fig. 1, the elution profile of H-2 alloantigenic activity measured by the indirect precipitation assay coincided with the anti-TL antiserum detection systems (data not shown). When, however, the crude papain-released RADA1 material was first pretreated with anti-H-2 serum and then applied to the Sephadex G-150 column, the TL antigenic activity detected by indirect precipitation was eluted in the usual position; but no H-2 antigenic activity was detected in that region. Thus, the papain-solubilized TL and H-2 antigen are present on different molecular fragments in the papain-solubilized form. As judged by their gel filtration behavior, the mol wt of the two antigens in this form is very similar, a conclusion consistent with the findings of Davies et al. (3) and of Vitetta et al. (9) for NP-40-solubilized TL antigens.

To further characterize the biochemical nature of the *Tla* gene product, we prepared [^{3}H]fucose and [^{14}C]amino acid-labeled TL antigen by a large scale indirect precipitation from the pooled TL eluate from the Sephadex G-150 column. The purity of the preparation was monitored, and the ratio of radioactivity in the specific precipitate to the control precipitate was greater than 10 to 1 in the case of the [^{3}H]fucose-labeled antigen and 2.5–1 for the [^{14}C]amino acid-labeled antigen. The doubly labeled TL antigen-antibody complex was dissolved in sodium dodecyl sulfate (SDS), reduced with β-mercaptoethanol, and analyzed by SDS–Sephadex G-150 column chromatography (Fig. 2). Both the [^{3}H]fucose label and [^{14}C]amino acid label were eluted together.

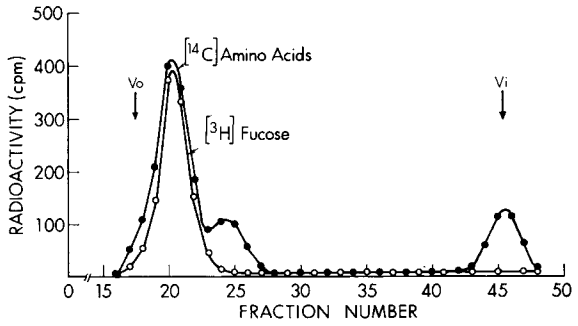

FIG. 2. SDS–Sephadex G-150 column chromatographic analysis of TL antigen doubly labeled with [^{3}H]fucose and [^{14}C]amino acids. The TL sample was a specific antibody precipitate prepared from papain-solubilized antigen ([^{3}H]fucose and [^{14}C]amino acid labeled) as described under Materials and Methods. The precipitate (approx. 2,500 cpm ^{14}C, 2,200 cpm ^{3}H) was dissolved in 1 ml of a 1% SDS, 0.15 M NaCl, 3% β-mercaptoethanol by boiling 1 min before application to the 80 × 0.9 cm column of Sephadex G-150. The column was eluted with 0.5% SDS–0.15 M NaCl, pH 7.4, buffer and 2.0-ml fractions were collected. The [^{14}C]amino acid pattern (●—●) and [^{3}H]fucose pattern (○—○) show coincidence of the major peak in the SDS solvent and thus support the conclusion that the antigen is a glycoprotein.

In order to estimate the approximate molecular size of the TL antigen, a [^{3}H]fucose-labeled TL antigen-antibody precipitate was mixed with a [^{14}C]-amino acid-labeled immunoglobulin preparation and subjected to chromatography (Fig. 3) under the same conditions as for the experiment in Fig. 2, except that the fractions contained small volumes. In Fig. 3*A*, it is seen that the fucose-labeled TL antigen eluted between the peaks for the heavy chain and light chain of the immunoglobulin standard. This position is identical to the elution position of tritiated fucose-labeled H-2.4 antigen isolated from the same papain digest and shown for comparison in part *B* of Fig. 3.

The data in Figs. 2 and 3 show that both [^{3}H]fucose and [^{14}C]amino acid are part of the TL alloantigen and hence establish that the antigen in a form isolated by anti-TL antibody is a glycoprotein. Also, the molecular size of the papain-digested fragment of the TL alloantigen is shown to be approximately

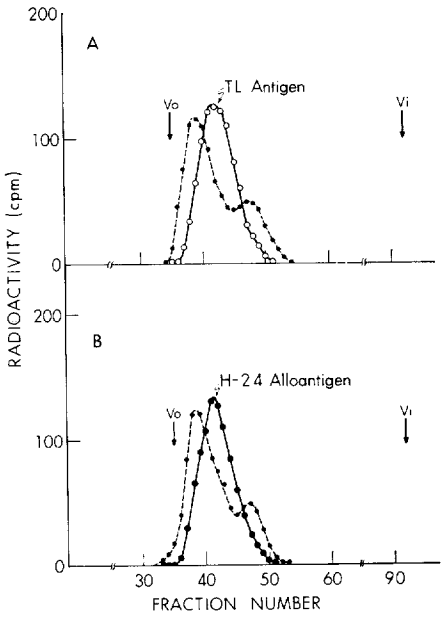

FIG. 3. SDS-Sephadex G-150 column chromatographic comparison of the TL antigen and H-2.4 antigen. Samples of [^{3}H]fucose-labeled TL antigen or [^{3}H]fucose-labeled H-2.4 alloantigen prepared by the indirect precipitation method were dissolved in 1 ml of 1% SDS in 0.15 M NaCl, 3% β-mercaptoethanol, mixed with [^{14}C]leucine-labeled IgG (from the myeloma cell MPC-11), and boiled at 100°C for 1 min. The samples were then applied to a column of Sephadex G-150 (0.9 × 90 cm), which was equilibrated and eluted with 0.5% SDS in 0.15 M NaCl, containing 0.01 M Tris-HCl, pH 8.4. Fractions of 0.8 ml were collected. Arrows indicate the elution position of standard substances, blue dextran and free fucose. (*A*) shows TL antigen profile (○—○) and IgG profile (●- - -●). The peak of heavy chain from the myeloma is at tube 39, light chain from the myeloma at 47. (*B*) shows the [^{3}H]fucose-labeled H-2a (H-2.4) antigen (○—○) and heavy and light chain profiles (●- - -●).

similar to that for the H-2.4 alloantigen fragment, which other studies[2] have shown to be approximately 38,000 in mol wt.

The carbohydrate moiety of the TL alloantigen was also analyzed further. [^{3}H]Fucose- and [^{14}C]amino acid-labeled TL alloantigen fragments complexed with antibodies were extensively digested with pronase to remove most of the amino acid residues from the carbohydrate chains, and the digest was analyzed by Sephadex G-50 column chromatography (Fig. 4). The [^{3}H]fucose radioactivity was eluted between the included and excluded volumes, whereas the [^{14}C]amino acid radioactivity was eluted in the included volume. These findings further confirm that the TL alloantigen fragment is a glycoprotein because the [^{3}H]fucose and [^{14}C]amino acid peak coincided during SDS–Sephadex G-150

[2] Schwartz, B. D., K. Kato, S. Cullen, and S. G. Nathenson. 1973. H-2 histocompatibility alloantigens: some properties of the molecules solubilized by NP-40. *Biochemistry.* In press.

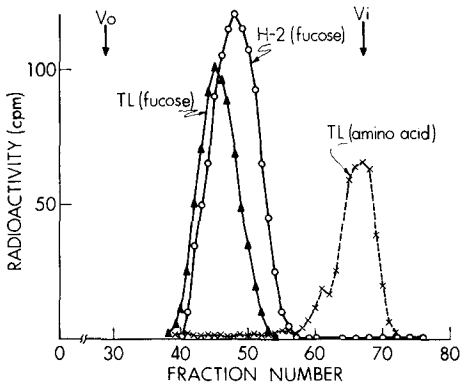

FIG. 4. Analysis of TL and H-2 glycopeptides by Sephadex G-50 column chromatography. [^{3}H]Fucose (▲—▲) and [^{14}C]amino acid (×- - -×) labeled TL antigen prepared by indirect precipitation was suspended in 2 ml of 0.01 M Tris-HCl buffer, pH 8.4, containing 0.15 M NaCl and 0.01 M $CaCl_2$ and digested with a total of 15 mg of pronase for 72 h as described previously (6). The digest was lyophilized, dissolved in 0.7 ml of water, and applied to a Sephadex G-50 fine column (0.9 × 90 cm), which was equilibrated and eluted with 0.01 M Tris-HCl, pH 8.4, containing 0.15 M NaCl. 0.9-ml fractions were collected. For comparison the pattern of the [^{3}H]fucose-labeled glycopeptide from the H-2.4 alloantigen (prepared by indirect precipitation) from a separate column run is plotted on the same graph. The TL glycopeptide pattern (▲—▲), which shows a peak corresponding to a mol wt of 4,500, is different from the H-2 glycopeptide pattern (○—○), which shows a peak corresponding to a mol wt of 3,500.

column chromatography, but moved separately as low mol wt materials after the pronase digestion had converted the high mol wt protein moiety to amino acids and peptides.

The [^{3}H]fucose-labeled glycopeptide from the TL alloantigen and that from the H-2 alloantigen (H-2.4) showed different elution behavior during the Sephadex G-50 column chromatography (Fig. 4). By calibrating the G-50 column with reference glycopeptide standards, we estimated the approximate mol wt of the peak of the TL glycopeptide to be approximately 4,500 daltons and that of the H-2 glycopeptide as 3,500 daltons, the latter being in agreement with our previous results (6).

We have established in these studies that the TL antigen can be solubilized from cell membranes by papain digestion, and that when isolated by indirect precipitation this soluble fragment is a glycoprotein of molecular size similar to the H-2 glycoprotein fragment solubilized by papain. Analysis of the glycopeptide portion of the TL product shows its mol wt to be slightly larger (4,500) than the H-2 glycopeptide (3,500). The differential size of the H-2 and TL alloantigen glycopeptides is most certainly explained by a differential carbohydrate structure because the condition of the pronase digestion employed is so severe that it allows only a few amino acids to remain still attached to the glycopeptides (6), although a difference in amino acid residues is not entirely excluded.

Our findings thus suggest the chemical similarity of the TL and H-2 antigens,

a similarity that has been noted previously (3); but the results also show distinct molecular differences in terms of their carbohydrate structure. Further studies on the chemical structure of these two genetically linked alloantigens should give information as to their possible relationship in terms of their genetic evolution and possibly their relationship and function on the cell surface.

SUMMARY

Thymus leukemia (TL) alloantigenic activity was solubilized by papain proteolytic digestion from intact RADA1 tumor cells. If the cells were labeled with amino acids and fucose, the TL alloantigen could be isolated as a doubly labeled glycoprotein fragment by indirect precipitation from the papain digest. This TL glycoprotein fragment was approximately the same mol wt as the papain-digested H-2.4 alloantigen fragment as judged by chromatography on Sephadex G-150 in sodium dodecyl sulfate. The carbohydrate chain of the TL glycoprotein obtained by exhaustive pronase digestion behaved as a glycopeptide of approximately 4,500 mol wt, as compared with the glycopeptide of the H-2.4 alloantigen that had a mol wt of about 3,500. Thus, the TL alloantigen can be solubilized by papain digestion as a glycoprotein fragment similar in mol wt to the H-2 alloantigen glycoprotein fragment. The carbohydrate chain of the TL glycoprotein is larger than the H-2 carbohydrate chain.

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