IMMUNOCHEMICAL ANALYSIS OF THE RELEASED LEU-2 (T8) MOLECULE

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Recent developments in hybridoma technology and T cell cloning have permitted more precise study of human T cell differentiation. Monoclonal antibodies have revealed several cell surface antigens that are important not only as T cell differentiation markers but also as T cell function–associated structures (1–4). One of these antigens, Leu-2 (T8), has been suggested to serve as a receptor for human class I major histocompatibility complex (MHC)[1] molecules (1, 4). Biochemical analysis of this molecule has revealed a unique structure. Leu-2 (T8) has been reported to be a heterodimer when isolated from thymocytes (5, 6), but a homodimer when isolated from perpherial blood T cells or from HPB-ALL cells (7, 8).

Consistent with these observations, our recent study demonstrated the homodimeric form of the Leu-2 (T8) antigen on HPB-ALL cells (9). We also have reported the development of enzyme-linked immunosorbent assays (ELISA) for the detection of the Leu-1, Leu-2, and Leu-3 antigens in their soluble forms. Using these assays, we have found that one antigen, Leu-2, is specifically and spontaneously released from T cells. The preliminary study comparing the released form of Leu-2 (RLeu-2) to the cellular form of Leu-2 (CLeu-2) led us to the conclusion that a 27,000-mol wt single chain form of Leu-2 antigen carrying only one Leu-2a determinant was released from Leu-2 positive T cells.

Since RLeu-2 has unique characteristics, as compared with CLeu-2, and since Leu-2 has been thought to be an important molecule in cellular interaction and MHC restriction, further study of the biological and immunochemical nature of this release was appropriate. In this report we describe the immmunochemical analysis of RLeu-2. We show that RLeu-2 and CLeu-2 are structurally related and that the released form is derived directly from the cell surface.

Materials and Methods

Monoclonal Antibodies. The mouse monoclonal anti–Leu-2a and anti–Leu-2b have been described previously (2). Purified preparations of these antibodies were obtained from Becton Dickinson, Mountain View, CA. Other monoclonal antibodies, 1D12 (mouse IgG1 anti-human mu chain) and L243 (mouse IgG2a anti-HLA-DR), were used as controls.

[1] *Abbreviations used in this paper:* CLeu-2, cellular Leu-2 antigen; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IEF, isoelectric focusing; 2ME, 2-mercaptoethanol; MHC, major histocompatibility complex; mol wt, molecular weight; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RLeu-2, released Leu-2 antigen; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Biosynthetic Labeling of HPB-ALL Cells. HPB-ALL cells were washed three times with phosphate-buffered saline (PBS). $1-2 \times 10^8$ cells were then suspended in 50 ml of RPMI medium lacking tyrosine, leucine, and phenylalanine, and supplemented with 5% dialyzed fetal calf serum (FCS); and incubated for 45 min at 37°C to deplete pools of intracellular amino acids. Then [^{3}H]tyrosine, [^{3}H]leucine, and [^{3}H]phenylalanine (1 mCi each) (New England Nuclear, Boston, MA) were added to the culture and the cells were incubated for 20 h at 37°C in 5% CO_2. After incubation, the supernatant was collected by centrifugation. Phenylmethylsulfonylfluoride (PMSF) (Sigma Chemical Co., St. Louis, MO) was added at a concentration of 1 mM. Pelleted cells were washed three times with PBS and a cell lysate was made with Nonidet P-40 (NP-40) as described previously (9).

Cell Surface Radioiodination and Incubation of Radiolabeled Cells. HPB-ALL cells were labeled with $Na^{125}I$ (Amersham, Arlington Heights, IL), 1 mCi per 1×10^7 cells, by the lactoperoxidase method (10). After washing the radiolabeled cells once with PBS, viable cells were collected by Ficoll-Hypaque gradient centrifugation. The viable cells at the interface were washed three times with PBS and resuspended in RPMI-1640 medium supplemented with 15% FCS, penicillin-streptomycin (20 ml of medium per 1×10^7 cells) and incubated for 18 h at 37°C. After incubation, supernatant and cell lysate were prepared as described above.

Immunoprecipitation and Gel Electrophoresis. Before specific immunoprecipitation, the samples were precleared by incubation with an irrelevant mouse IgG1 coupled to Sepharose 4B beads (Pharmacia, Uppsala, Sweden) at 4°C. After removing the beads by centrifugation, the samples were divided into aliquots and specific monoclonal antibody–coupled Sepharose 4B beads (25 μl of packed wet volume containing 25 μg of antibody) were added and incubated at 4°C. The beads were then washed four times with 0.2 M Tris-HCl, pH 8.0, 0.5 M NaCl, 0.5% NP-40, 0.2 mM PMSF buffer. In the immunoprecipitations where anti-Leu-2b was used, 25 μg of anti-Leu-2b and 25 μl of Protein A–coupled Sepharose beads (Pharmacia) were added to the sample at the same time and were incubated in the same manner described above. Immunoprecipitated materials were eluted from the beads with either SDS sample buffer with 2-mercaptoethanol (2ME) (11) or with isoelectric focusing sample buffer with 2ME (12), and analyzed either by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or by two-dimensional gel techniques using non-equilibrium pH gel electrophoresis and SDS-PAGE.

Peptide Mapping Analysis. Peptide mapping analysis on one-dimensional SDS-PAGE was performed according to the method described by Cleveland et al. (13). Metabolically labeled or radioiodinated CLeu-2 and RLeu-2 were digested with *Staphylococcus aureus* V8 protease (Miles Laboratories, Elkhart, IN) at a concentration of 50 μg per ml (100 μl of each sample) and fragments analyzed on a 15% polyacrylamide gel.

Results

Molecular Weight of RLeu-2. In our previous experiments (9), we estimated the molecular weight of RLeu-2 to be 27,000 by iodination of the molecule after affinity isolation. We used two other methods to confirm the mol wt of RLeu-2; metabolic labeling and cell surface radioiodination preceding cell culture. With respect to metabolic labeling, HPB-ALL cells were labeled with radioactive amino acids overnight. Cell viability was >90% after incubation. Both the culture supernatant and the cell lysate were subjected to immunoprecipitation. As shown in Fig. 1, a specific band of 27,000 mol wt was identified in the Leu-2 precipitate made from the culture supernatant (lane *C*), whereas a 33,000 mol wt band was seen in the Leu-2 precipitate from cell lysate (lane *A*).

Following cell surface radioiodination, HPB-ALL cells were cultured overnight. After incubation, cell viability was >95%. Both the culture supernatant and the cell lysates from before and after incubation were subjected to immu-
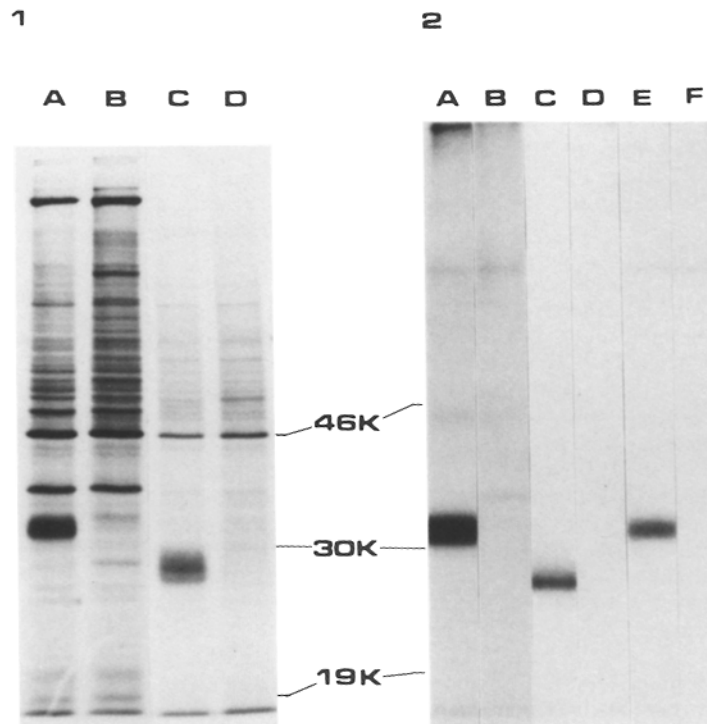
FIGURE 1.   Spontaneous release of the metabolically labeled Leu-2 antigen from HPB-ALL cells. Antigen was immunoprecipitated from either metabolically labeled HPB-ALL cell lysate (A and B) or from culture supernatant (C and D), and analyzed on 10% SDS-PAGE under reducing conditions. Anti–Leu-2a Sepharose 4B beads (A and C) and 1D12-Sepharose 4B beads (B and D) were used for immunoprecipitations.

FIGURE 2.   Spontaneous release of the cell surface radiolabeled Leu-2 antigen from HPB-ALL cells. Antigen was immunoprecipitated from either cell surface radioiodinated HPB-ALL cell lysate (A and B) or from culture supernatant (C and D) and analyzed on 10% SDS-PAGE under reducing conditions. Radioiodinated cell lysate prepared after incubation (E and F) was also analyzed. Immunoprecipitations were performed with anti–Leu-2a-Sepharose 4B beads (A, C and E) and 1D12-Sepharose 4B beads (B, D, and F).

noprecipitation. Fig. 2 shows that a 27,000-mol wt Leu-2 chain accumulated in the supernatant (lane C). Leu-2 antigen precipitated from cell lysates prepared before incubation appeared as a 33,000-mol wt polypeptide chain as was seen previously (Fig. 2A) (9). Residual intact cellular Leu-2 was found in the cell lysate prepared after incubation (Fig. 2E), but in reduced quantity. In experiments where anti–Leu-1 or anti–Leu-3a beads were used in control immunoprecipitations from the supernatant, no observable band was seen for either Leu-1 or Leu-3 antigen (data not shown). Therefore, it is unlikely that the radioactive Leu-2 antigen in the supernatant was derived from dead cells. These two experiments indicated that RLeu-2 antigen found in the supernatant of HPB-ALL is derived directly and specifically from CLeu-2 on the cell surface.

RLeu-2 and CLeu-2 Have Different Appearances in Two-dimensional Gel Analysis.   RLeu-2 and CLeu-2 were compared by two-dimensional gel analysis (Fig. 3). CLeu-2 appeared as a smear in a wide range of pH, although the majority of it was distributed in the basic area (Fig. 3A). On the other hand, RLeu-2 appeared
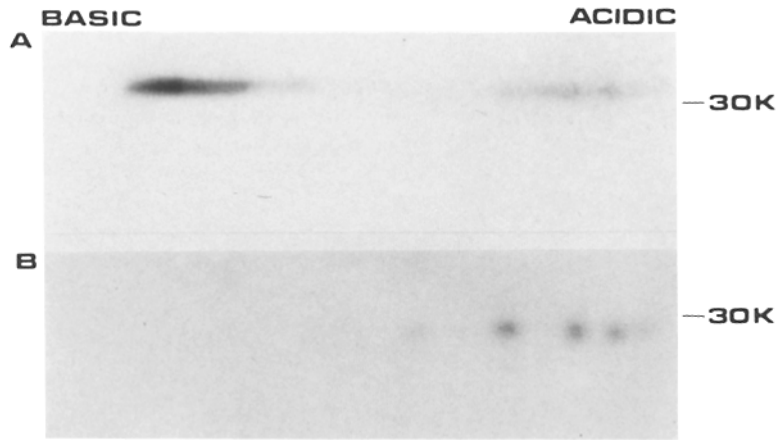
FIGURE 3. Two-dimensional analysis of CLeu-2 and RLeu-2. Radioiodinated cell surface Leu-2 (A) and released Leu-2 (B) were precipitated by anti–Leu-2a Sepharose 4B and were analyzed by reducing two-dimensional gel analysis (nonequilibrium pH gel electrophoresis and 10% SDS-PAGE).

as several distinct spots in the more acidic area showing less charge heterogeneity than CLeu-2 (Fig. 3 B).

RLeu-2 and CLeu-2 Share More Than One Antigenic Determinant. Our previous results have demonstrated that RLeu-2 and CLeu-2 share the Leu-2a determinant. Since RLeu-2 and CLeu-2 had quite different immunochemical characteristics, it was important to know whether simple cross-reactivity accounted for the anti–Leu2a binding to RLeu-2. At least two different antigenic determinants, Leu-2a and Leu-2b, have been identified on the Leu-2 molecule by monoclonal antibodies. Possession of both the Leu-2a and Leu-2b determinants by RLeu-2 would make unrelated cross-reactivity of the RLeu-2 and CLeu-2 molecules highly unlikely. Using anti–Leu-2a and anti–Leu-2b, we explored the antigenic makeup of RLeu-2 and CLeu-2. Fig. 4-1 demonstrates that both anti–Leu-2a (lane A) and anti–Leu-2b (lane C) precipitated the CLeu-2. Similarly, Fig. 4-2 shows that both anti–Leu-2a (lane A) and anti–Leu-2b (lane C) precipitated RLeu-2, suggesting that RLeu-2 contained both antigenic determinants. RLeu-2 possession of both Leu-2a and Leu-2b epitopes was confirmed by sequential immunoprecipitation experiments. Pretreatment of RLeu-2–containing supernatant with anti–Leu-2a totally removed all material immunoreactive not only with anti–Leu-2a (Fig. 4-2, lane E) but also with anti–Leu-2b (Fig. 4-2, lane F). Pretreatment of the supernatant with a class-matched irrelevant monoclonal antibody removed neither the anti–Leu 2a (Fig. 4-2, lane G) nor the anti–Leu-2b (Fig. 4-2, lane H) immunoprecipitable material. Therefore, the Leu-2a and Leu-2b antigenic determinants co-exist on the single polypeptide chain of RLeu-2.

Peptide Mapping Analysis of RLeu-2 and CLeu-2. Futher structural comparison was made by peptide analysis following limited proteolysis (Fig. 5). Radioiodinated RLeu-2 and CLeu-2 were cleaved into their peptide fragments by *Staphylococcus aureus* V8 protease and those fragments were analyzed on a 15% polyacrylamide gel. CLeu-2 (Fig. 5 A) and RLeu-2 (Fig. 5 B) produced almost
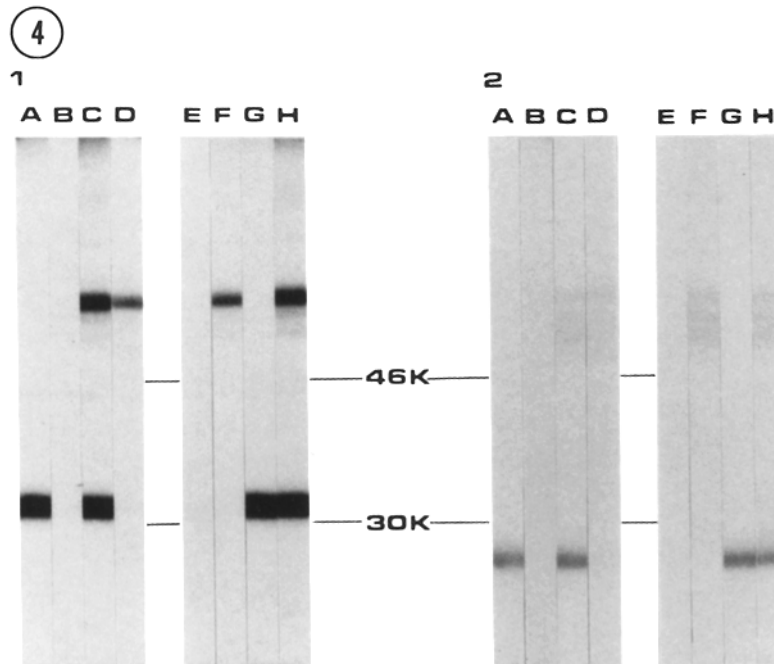
FIGURE 4.   Sequential immunoprecipitation analysis of CLeu-2 (4-1) and RLeu-2 (4-2). Radioiodinated Leu-2 antigens were precipitated by anti–Leu 2a Sepharose 4B beads (A) or by anti–Leu-2b-Protein-A Sepharose 4B beads (C) from radioiodinated cell lysates (4-1) or from culture supernatants (4-2) of HPB-ALL cells. For sequential immunoprecipitation experiments, cell lysates or culture supernatants were precleared by either anti–Leu-2a Sepharose 4B (E and F) or 1D12 Sepharose 4B (G and H). The residual materials were divided into aliquots and were used for the next immunoprecipitations with anti–Leu-2a Sepharose 4B (E and G) or with anti–Leu-2b-Protein A Sepharose 4B (F and H). 1D12 Sepharose 4B (B) and L243-Protein-A-Sepharose 4 (D) were used as controls for anti-Leu-2a Sepharose 4B and anti–Leu-2b Protein-A Sepharose, respectively.

identical peptide maps, suggesting significant homology between RLeu-2 and CLeu-2. A peptide map of CLeu-2 metabolically labeled with three radioactive amino acids was made to examine whether new fragments would be produced. As is shown in Fig. 5C, metabolically labeled CLeu-2 produced peptide fragments, identical to those of radioiodinated RLeu-2 and CLeu-2, indicating that all the fragments had been identified by ^{125}I tyrosine labeling and confirming the structural homology of the two forms of the Leu-2 antigen.

Discussion

In an earlier study (9), we discovered the Leu-2 molecule in supernatants of cells bearing Leu-2 on their surfaces. We demonstrated that the released form of Leu-2 (RLeu-2) and the cellular form of Leu-2 (CLeu-2) had different structures. First, RLeu-2 had a single Leu-2a determinant, while CLeu-2 had two or more Leu-2a determinants. Secondly, radioiodinated RLeu-2 was a single polypeptide chain of 27,000 mol wt under both reducing and nonreducing conditions, while radioiodinated CLeu-2 was a homodimeric structure consisting
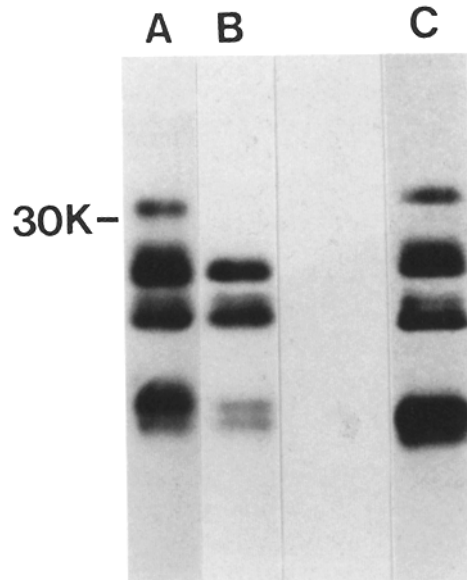
FIGURE 5.   Peptide mapping analysis of CLeu-2 and RLeu-2. Radioiodinated CLeu-2 (A)
RLeu-2 (B) were digested with Staphylococcus aureus V8 protease and analyzed on 15% SDS-
PAGE. Peptide mapping of metabolically labeled CLeu-2 (C) was also shown for comparison.

of disulfide-linked 33,000-mol wt subunits. The results reported here further
extend the comparison of RLeu-2 and CLeu-2.

RLeu-2 and CLeu-2 are similar in several respects. First, they share at least
two, and probably more, antigenic determinants, as demonstrated by similar
detection in Leu-2 ELISA and depletion in sequential immunoprecipitation
studies. Secondly, RLeu-2 is a product of CLeu-2. In biosynthetic labeling studies,
RLeu-2 appearance in cell supernatant was accompanied by decreasing amounts
of CLeu-2 (unpublished observations). In studies where viable cells were surface-
radioiodinated and then incubated overnight, RLeu-2 appeared spontaneously
in the culture supernatant. These studies confirmed that RLeu-2 was not an
artefact of the previously used labeling or isolation procedures. Finally, peptide
maps of RLeu-2 and CLeu-2 demonstrated marked similarity, suggesting struc-
tural homology between the two species.

RLeu-2 and CLeu-2 also possess interesting differences. In two-dimensional
gel analysis, CLeu-2 and RLeu-2 showed quite different patterns. Although
CLeu-2 appeared as a smear over a wide range of pH of which the major part
was in the basic portion of the gel, RLeu-2 appeared as several spots having less
charge heterogeneity in the more acidic portion.

Lyt2-Lyt3, the Leu-2 homologous structure in the mouse (14), has been
characterized by many investigators. In many cases Lyt2-Lyt3 was found as a
dimer or a tetramer bound by disulfide bonds (15–18). However, a few reports
suggested the presence of the free single chain of Lyt-2–Lyt-3 antigen under
nonreducing conditions (19, 20). Like Lyt-2–Lyt-3, Leu-2 (T8) has subunits
joined by disulfide bonds (5–9). Under reducing conditions, Leu-2 from thy-
mocytes separates into two subunits of 43,000 and 32,000 mol wt (5). From

HPB-ALL cells, only the 33,000 (7–9) mol wt chain has been identified using anti–Leu-2a, OKT5, or OKT8. Under nonreducing conditions, no free subunit of Leu-2 (T8) has been identified. In our studies, cell surface radioiodinated Leu-2 and metabolically labeled Leu-2 from HPB-ALL cells showed identical two-dimensional gel patterns. Furthermore, no free subunit of metabolically labeled Leu-2 was found under nonreducing conditions (unpublished observations). Therefore, on HPB-ALL cells it is likely that Leu-2 antigen is composed of identical disulfide chains bonded to each other and that no free subunit is present.

RLeu-2 is a single chain and has a mol wt of 27,000. It appears to arise spontaneously from CLeu-2 by some mechanism intrinsic to the lymphocyte. Production of single chains from a disulfide-bonded macromolecule requires either reduction of the disulfide bonds or cleavage of the peptide at a specific site. Since Leu-2 (T8) has a membranous hydrophobic portion (8), it may be speculated that the extracellular portions of the Leu-2 (T8) are cleaved to form RLeu-2, leaving hydrophobic fragments behind on the cell membrane. The specific cleavage envisioned here suggests the presence of a cell surface enzyme.

Leu-2 (T8) has been associated with recognition of class I MHC molecules (1, 4, 21). Its release suggests some immunoregulatory role for RLeu-2. Lyt-2–Lyt-3 is not known to be released. However, class I H-2–related molecules recently were found in mouse serum (22, 23). These H-2 molecules secreted from mouse liver have a mol wt of 40,000, which is smaller than that of the classical class I H-2 molecule (23). The secretion mechanism of the truncated H-2 antigen appears significantly different from that of the release of the Leu-2 antigen. However, the release of members of this important immunological recognition system in mouse and man may point to the existence of a previously unrecognized immunoregulatory mechanism.

Summary

We recently have found that the human T cell antigen Leu-2 was specifically released from Leu-2–bearing cells. The preliminary study showed that the released Leu-2 (RLeu-2) from HPB-ALL cells was composed of a single polypeptide chain of 27,000 molecular weight (mol wt), which was smaller than the subunit of the homodimeric molecule found on the cell surface. In the present study, RLeu-2 was further characterized and compared with cellular Leu-2 (CLeu-2). Metabolically radiolabeled Leu-2 was released from HPB-ALL cells and this released Leu-2 molecule had a mol wt of 27,000. Cell surface radioiodinated HPB-ALL cells were found to release radioactive Leu-2 molecules and this antigen also had the same mol wt of 27,000. In both experiments, the CLeu-2 was reconfirmed to be composed of a 33,000-mol wt subunit under reducing conditions. These experiments establish that the 27,000-mol wt single polypeptide chain of Leu-2 released from the cell is derived directly from the homodimeric Leu-2 molecule on the cell surface, presumably by a specific proteolytic cleavage.

Two-dimensional gel analysis showed that CLeu-2 exhibited extensive charge heterogeneity with predominantly basic isoelectric points, whereas RLeu-2 was a group of more acidic proteins with less charge heterogeneity. Although CLeu-

2 and RLeu-2 showed several different immunochemical characteristics, the homology between these two antigens was confirmed by the following results: CLeu-2 and RLeu-2 were found to share at least three different antigenic determinants, Leu-2a and Leu-2b, and those which were detected by a polyvalent rabbit antiserum. Significant similarities between CLeu-2 and RLeu-2 were demonstrated by peptide mapping analysis of these antigens. Therefore, RLeu-2 appears to be the specific, physiological product of the CLeu-2 protein.

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