# Lyt-2 AND Lyt-3 ANTIGENS ARE ON TWO DIFFERENT POLYPEPTIDE SUBUNITS LINKED BY DISULFIDE BONDS

## Relationship of Subunits to T Cell Cytolytic Activity*

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The Lyt-2 and Lyt-3 antigens in the mouse are selectively expressed on a subpopulation of T lymphocytes that include the cytotoxic effectors and suppressors (1–3). Two separate alleles of each antigen are known, i.e., Lyt-2.1, Lyt-2.2, Lyt-3.1, and Lyt-3.2. Genes controlling the expression of the Lyt-2 and Lyt-3 antigens are closely linked to each other on chromosome 6 (4), and are also closely linked to the $\kappa$ light chain locus (5–7).

Because of this close association with an immunoglobulin locus and their selective expression on functionally distinct subpopulations, the macromolecule(s) carrying the Lyt-2 and Lyt-3 antigens have been thought to perform essential functions, most likely involving cellular interactions or recognition of antigen. Support for this idea has come from functional studies of cytotoxic effector T cells, in which antibodies to Lyt-2 or Lyt-3 were shown to block killing by binding to the effector cells (8, 9). Antibodies to Lyt-2 and Lyt-3 also block allogeneic sitmulation of T cells in mixed lymphocyte culture (10, 11).

It is important to understand the structure of the molecular species carrying the Lyt-2 and Lyt-3 antigens in order to analyze its function. The original immunoprecipitation studies with either Lyt-2 or Lyt-3 antibodies from surface $^{125}$I-labeled thymocytes showed protein(s) of ~35,000 $M_r$ under reducing conditions (12). In preclearing experiments (13), it was concluded that Lyt-2 and Lyt-3 antigens reside on separate molecules, although an association between the two molecules was apparent. Our recent studies of Lyt-2 from thymocytes using a monoclonal antibody and two-dimensional gel electrophoresis run under reducing conditions showed two proteins with a basic charge and molecular weights between 30,000–38,000 (14). These two proteins were associated through disulfide bonds into larger forms (65,000–75,000 and 140,000 $M_r$) under nonreducing conditions.

We show here that Lyt-2 and Lyt-3 antigens are present on individual polypeptide subunits that are disulfide-bonded into multimeric forms on the cell surface. We also show that the Lyt-3 subunit can be selectively cleaved from viable cells with low levels of trypsin with a concommitant but not parallel loss of cytolytic effector cell activity. We use monoclonal antibody blocking of cytotoxic effector cell activity and

* Supported in part by grants CA-04681 and GM-17367 from the National Institutes of Health, and by the Veterans Administration.

‡ Recipient of National Institutes of Health Fellowship 1F32 CA-06207.

selective protease removal of Lyt-3 to investigate the role of the Lyt-2, Lyt-3 molecule in T cell recognition of target cell antigens. Further, by examining heterozygotes we find that neither Lyt-2 nor Lyt-3 is allelically excluded in expression on T cells. We conclude that the Lyt-2, Lyt-3 molecules are closely associated with but not identical to cytolytic T cell antigen receptors.

## Materials and Methods

*Antibodies.* Monoclonal rat anti-mouse Lyt-2 (53-6.7) and rat anti-mouse Lyt-3 (53-5) have been previously described (14, 15). Antibodies were purified from culture supernate on goat anti-rat Ig-Sepharose columns. Monoclonal anti-Lyt-2.2 (19/178) was a kind gift of Dr. U. Hämmerling, Memorial Sloan-Kettering Cancer Center, New York. This antibody was used as serum from hybridoma-bearing mice without further purification.

*Mice.* Young BALB/cNHz mice (6–8 wk) used for the biochemical studies were bred at Stanford University, Stanford, Calif. C57BL/6 and DBA/2 mice used for the cytotoxicity studies were obtained from Simonsen Laboratories, Gilroy, Calif.

*Cell Surface Labeling and Detergent Extraction.* BALB/cNHz thymocytes or lymph node cells were labeled with $^{125}$I by a modification of the lactoperoxidase technique. To $1 \times 10^8$ cells in 1 ml phosphate-buffered saline (PBS)[1] at 20°C were added 1.0 mCi $^{125}$I (Amersham Corp., Arlington Heights, Ill.), 25 μg lactoperoxidase (B grade; Calbiochem-Behring Corp., La Jolla, Calif.), and successive 10-μl pulses of $H_2O_2$ (0.3 mM, 1 mM, 3 mM, and 9 mM) at 5-min intervals. Cells were washed twice in PBS and extracted in lysis buffer (0.5% Nonidet-P40 [NP-40, Particle Data Laboratories, Ltd., Elmhurst, Ill.]; 50 mM Tris; 150 mM NaCl; 0.02% $NaN_3$; 5 mM EDTA; 50 μM phenylmethylsulfonyl fluoride; 0.2 TIU/ml Aprotinin [Sigma Chemical Co., St. Louis, Mo.]; 1 μg/ml pepstatin A [Sigma Chemical Co.]; and 50 mM iodoacetamide, pH 8.0) for 30 min at 4°C. Nuclei were removed by centrifugation at 5,000 *g* for 20 min.

*Immunoprecipitation and Gel Electrophoresis.* Immunoprecipitations were with ~5 μg antibody per extract from $2 \times 10^7$ cells. After a 1-h incubation on ice, antigen-antibody complexes were collected by the addition of 10 μl of 10% fixed *Staphylococcus aureus* (staph A) Cowan I strain (Tufts New England Enzyme Center, Boston, Mass.). For the rat antibodies that do not bind to protein A directly, the staph A was precoated with affinity-purified mouse (SJL/J) anti-rat Ig and washed twice before addition to the cell extract. After 45 min on ice, the staph A-antibody-antigen complexes were washed three times in washing buffer (0.5% NP-40, 0.45 M NaCl, 50 mM Tris, 5 mM KI, and 0.02% $NaN_3$, pH 8.3), and then extracted with sample buffer for gel electrophoresis. Immunoprecipitates were analyzed either by (*a*) 10% one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 16); (*b*) 10% SDS-PAGE using N,N'-diallyltartardiamide (DATD) cross-linked instead of bis-acrylamide (17); or (*c*) two-dimensional gels with a nonequilibrium charge separation in the first dimension (18), using a 4:1 ratio of pH 3.5–10 to pH 8–9.5 ampholines (LKB Western, Pleasant Hill, Calif.), and 10% SDS-PAGE in the second dimension. Molecular weight markers (Pharmacia Fine Chemicals, Piscataway, N. J.) run on each gel were visualized by staining with Coomassie Brilliant Blue. Autoradiography was with intensifying screens (Cronex Lightning-plus; DuPont Instruments, Wilmington, Del.) using Kodak X-Omat R film (Eastman Kodak Co., Rochester, N. Y.) at −70°C.

*T Cell Cytotoxicity.* Cytotoxic T cells were obtained by immunizing C57BL/6 male mice, aged 2–3 mo, with $3 \times 10^7$ P815 (DBA/2 mastocytoma) cells intraperitoneally. P815 was carried in vivo by intraperitoneal injection of $10^7$ cells into DBA/2 mice every 5–10 d. 11 d after immunization, spleen cells were used as the source of cytotoxic effectors. Cytotoxicity was assessed against $^{51}$Cr-labeled P815 target cells, labeled by incubating $10^7$ cells in 100 μCi $^{51}$Cr in 0.5 ml at 37°C for 1 h. Assays were in round-bottomed microtiter plates (Linbro Chemical Co., Hamden, Conn.), using 0.1 ml target cells ($2 \times 10^5$ cells/ml) and 0.1 ml spleen effector cells in varying concentration to give spleen cell:target ratios of 100:1, 50:1, 25:1, 12:1, and

[1] *Abbreviations used in this paper:* DATD, diallyltartardiamide; FACS, fluorescence-activated cell sorter; NP-40, Nonidet-P40; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

6:1, with triplicate samples of each concentration. The cell mixture was pelleted by centrifugation at 100 *g* for 3 min and incubated for 4 h at 37°C in 5% $CO_2$. After centrifugation at 600 *g* for 10 min, 0.1 ml of the supernate was removed for determination of $^{51}$Cr released. Cytotoxicity was determined as previously described (19). Killing is expressed as lytic units per $10^7$ spleen cells. One lytic unit is defined as the number of spleen cells necessary to specifically lyse 40% of the target cells. Triplicate killing curves were derived for each cell treatment. For trypsinization of effector cells before the assay, spleen cells were collected and washed in PBS without fetal calf serum, and 0.5-ml aliquots containing $2 \times 10^7$ cells were incubated with varying amounts of trypsin [L(tosylamide-2-phenyl)-ethyl-chloromethyl ketone trypsin; Worthington Biochemical Corp., Freehold, N. J.] for 10 min at 37°C. The reaction was stopped by the addition of 10 ml PBS containing 10% fetal calf serum. The cells were washed twice and then used as effectors.

*Blocking of Cytotoxicity by Monoclonal Antibodies.* Spleen effector cells at $2 \times 10^7$ cells/ml were incubated with 20 μg monoclonal antibody for 30 min on ice, followed by the addition of an equal volume of medium containing SJL/J anti-rat Ig at 1:25 dilution. After another 30 min on ice, the cells were diluted for use as effectors without washing.

## Results

*Specificities of the Antibodies.* Three monoclonal antibodies that each recognize a distinct Lyt-2 or Lyt-3 specificity were used in parallel studies for analysis of the macromolecular antigens. The reactivities of the anti-Lyt-2.2 (19/178) and the anti-Lyt-2 (53-6.7) antibodies were identical throughout and are consistent with a close spatial relationship of the two antigenic determinants. This was further supported by blocking studies, in which either antibody, if added initially to viable cells, showed complete blocking of the other (data not shown). A third antibody (53-5) was identified as reacting with an Lyt-3 specificity based upon studies that showed complete blocking of its binding after pretreatment of viable cells with anti-Lyt-3 alloantibodies (data not shown). This latter antibody (53-5) binds to cells from all strains examined so far, but shows a >200-fold higher affinity for Lyt-3.2-bearing cells than for Lyt-3.1-bearing cells (data not shown).

*Characteristics of the Lyt-2, Lyt-3 Macromolecule.* Immunoprecipitations with all three antibodies from NP-40 extracts of $^{125}$I-labeled thymocytes showed identical patterns on SDS-PAGE (Fig. 1). Under reducing gel conditions, distinct, heavily labeled proteins of 34,000 and 38,000 $M_r$ were seen. A third protein that migrated as a broad

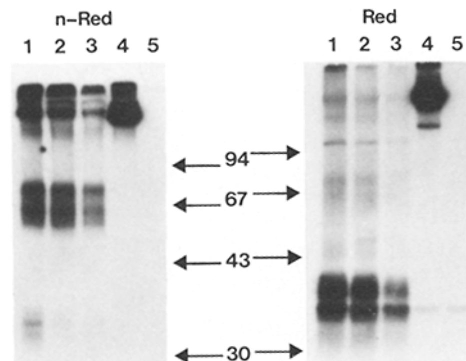


FIG. 1. Immunoprecipitation of Lyt-2 and Lyt-3 from surface $^{125}$I-labeled BALB/cNHz thymocytes. Immunoprecipitated proteins were electrophoresed on 10% polyacrylamide gels in the presence of SDS under both reducing and nonreducing conditions. Monoclonal antibodies used for immunoprecipitation were (1) anti-Lyt-2.2 (19/178); (2) anti-Lyt-2 (53-6.7); (3) anti-Lyt-3 (53-5); (4) anti-T200 (30-G12); (5) background (staph A alone).

band at 30,000 $M_r$ was also specifically precipitated, although this species was not labeled as heavily as the other two species. When analyzed under nonreducing gel conditions, each antibody specifically precipitated proteins of 140,000 and 65,000–75,000 $M_r$. The 65,000–75,000 $M_r$ protein(s) migrated as a broad band. This band is shown below to be composed of several kinds of dimers of the 30,000, 34,000, and 38,000 $M_r$ subunits seen under reducing conditions. In some experiments, the 30,000 $M_r$ subunit was seen under nonreducing gel conditions after immunoprecipitation with anti-Lyt-3, but not after immunoprecipitation with anti-Lyt-2 (Fig. 2). Under reducing gel conditions, the 30,000 $M_r$ subunit was seen after immunoprecipitation with both anti-Lyt-2 and anti-Lyt-3.

*Lyt-2 and Lyt-3 Antigens Are on the Same Macromolecule.* We used monoclonal anti-Lyt-2 and anti-Lyt-3 antibodies conjugated on Sepharose to show that both Lyt-2 and Lyt-3 antigens are on the same macromolecule. A detergent lysate from $^{125}$I-labeled thymocytes was divided into three equal portions that were (*a*) given no treatment, (*b*) passed through the anti-Lyt-2 absorbent, or (*c*) passed through the anti-Lyt-3 absorbent. Each portion was then subjected to immunoprecipitation with anti-Lyt-2.2, anti-Lyt-2, anti-Lyt-3, and anti-T200 (as a control). The results (Table I) showed that the anti-Lyt-2 absorbent removed reactivity with both anti-Lyt-2 and anti-Lyt-3 antibodies, but not with anti-T200. Similarly, the anti-Lyt-3 absorbent removed reactivity with anti-Lyt-3 but left some reactivity with anti-Lyt-2. Gel analysis of the Lyt-2$^+$, Lyt-3$^-$ material showed a 70,000 $M_r$ protein under nonreducing conditions that, under reducing conditions, ran as the 38,000 and 34,000 $M_r$ subunits (data not shown). These data show that the Lyt-2 and Lyt-3 antigens are co-expressed on the same macromolecules, but that a small amount of Lyt-2$^+$, Lyt-3$^-$ material exists in the detergent lysates.

*Lyt-2 and Lyt-3 Antigens Reside on Individual Subunits.* Lyt-2 and Lyt-3 antigens were identified on individual subunits by reduction of the molecule in detergent extracts (10 mM dithioerythritol, 3 h, 4°C) and alkylation (250 mM iodoacetamide) before immunoprecipitation with anti-Lyt-2 and anti-Lyt-3 antibodies. Both the anti-Lyt-2.2 and anti-Lyt-2 immunoprecipitated the 34,000 and 38,000 $M_r$ polypeptides but

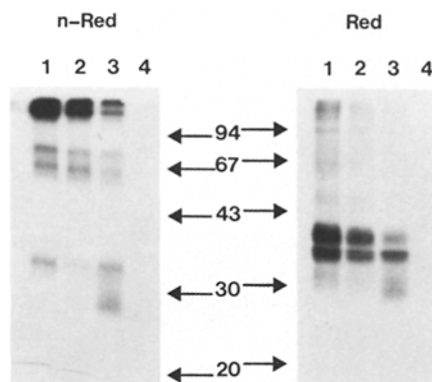


FIG. 2. Immunoprecipitation of Lyt-2 and Lyt-3 from surface $^{125}$I-labeled BALB/cNHz thymocytes. Immunoprecipitated proteins were electrophoresed on 10% polyacrylamide gels in the presence of SDS under both reducing and nonreducing conditions. Monoclonal antibodies used for immunoprecipitations were (1) anti-Lyt-2.2 (19/178); (2) anti-Lyt-2 (53-6.7); (3) anti-Lyt-3 (53-5); (4) background (staph A alone).

TABLE I
*Lyt-2 and Lyt-3 Antigens Are on the Same Macromolecule*

| Antibody | Immunoprecipitation* | | |
|---|---|---|---|
| | Nontreated lysate | Lyt-2-depleted lysate‡ | Lyt-3-depleted lysate§ |
| Anti-Lyt-2 (53-6.7) | 23 | 2.6 | 6.0 |
| Anti-Lyt-3 (53-5) | 16 | 4.4 | 2.8 |
| Anti-T200 (55-10.2) | 76 | 81 | 80 |
| — | 2.2 | 2.6 | 2.6 |

* Number are cpm in the immunoprecipitate ($\times 10^{-3}$) from surface $^{125}$I-labeled BALB/cNHz thymocyte lysates.

‡ The lysate was depleted of Lyt-2 by passage over an anti-Lyt-2 (53-6.7) antibody-Sepharose column.

§ The lysate was depleted of Lyt-3 by passage over an anti-Lyt-3 (53-5) antibody-Sepharose column.

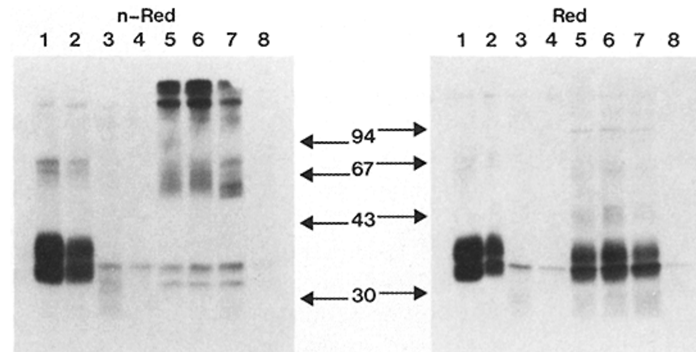


FIG. 3. Immunoprecipitation of anti-Lyt-2 and anti-Lyt-3 from surface $^{125}$I-labeled BALB/cNHz thymocytes after reduction and alkylation of the detergent lysate. Immunoprecipitated proteins were electrophoresed on 10% polyacrylamide gels in the presence of SDS under both reducing and nonreducing conditions. Lanes 1-4 are immunoprecipitated proteins from reduced and alkylated detergent lysate using monoclonal antibodies: (1) anti-Lyt-2.2 (19/178); (2) anti-Lyt-2 (53-6.7); (3) anti-Lyt-3 (53-5); and (4) background (staph A alone). Lanes 5-8 are immunoprecipitated proteins from an alkylated but not reduced detergent lysate using monoclonal antibodies: (5) anti-Lyt-2.2 (19/178); (6) anti-Lyt-2 (53-6.7); (7) anti-Lyt-3 (53-5); and (8) background (staph A alone).

not the 30,000 $M_r$ polypeptide, whereas the anti-Lyt-3 antibody reacted with the 30,000 $M_r$ polypeptide but not the 34,000 or 38,000 $M_r$ polypeptides (Fig. 3). These patterns of reactivity were the same whether the gels were run under reducing or nonreducing conditions, indicating that the initial reduction of the cell lysate was complete. Therefore, the Lyt-3 antigenic determinant (as identified by rat monoclonal antibody 53-5) is on the 30,000 $M_r$ subunit, whereas the Lyt-2.2 and Lyt-2 antigenic determinants (as identified by monoclonal antibodies 19/178 and 53-6.7) reside on either the 34,000 or 38,000 $M_r$ subunits or both. As noted above, small amounts of Lyt-$2^+$, Lyt-$3^-$ material was identified using immunoadsorbent columns. Similarly, some immunoprecipitation experiments revealed small amounts of free Lyt-3 subunit (30,000 $M_r$) in nonreduced detergent lysates (Fig. 2).

*Subunit Composition of the Lyt-2,Lyt-3 Macromolecule.* Two-dimensional SDS-PAGE of the Lyt-2,Lyt-3 macromolecule provided additional information about the subunit composition. An anti-Lyt-2 immunoprecipitate was electrophoresed under nonreduc-

ing conditions on 10% DATD cross-linked SDS tube gels, followed by electrophoresis in the second dimension under reducing conditions on 12.5% bisacrylamide-acrylamide slab gels (Fig. 4). This analysis showed that the 140,000-$M_r$ species seen under nonreducing conditions is composed of all three of the 38,000, 34,000, and 30,000 $M_r$ subunits. A larger macromolecule (>200,000 $M_r$) also reduced to the same three subunits, whereas the 65,000–75,000-$M_r$ material was composed of at least two kinds of molecules, including dimers of (*a*) the 38,000 and 30,000 $M_r$ species, and (*b*) the 34,000 and 30,000 $M_r$ species. The existence of 34,000–34,000, 38,000–38,000, and 38,000–34,000 $M_r$ dimers is not proven or disproven in Fig. 4. However, the Lyt-2$^+$,Lyt-3$^-$ material identified by use of immunoadsorbent columns (see above) consisted of only 38,000–34,000 $M_r$ dimers and not 38,000–38,000 or 34,000–34,000 $M_r$ dimers. Therefore, three of the six possible dimeric combinations of the 38,000, 34,000, and 30,000 $M_r$ subunits have been positively identified. Interestingly, these three combinations are all heterodimers (dimers of nonidentical subunits).

*Two-Dimensional (Charge vs. Size) Separation of Lyt-2,Lyt-3 Macromolecules from Thymocytes.* Two-dimensional gels of an Lyt-2 immunoprecipitate run under reducing conditions showed that the three subunits run with very basic charges (Fig. 5).The Lyt-3 subunit (30,000 $M_r$) is the most basic species and was not seen in earlier two-dimensional gel experiments (14) where it had migrated off the basic end of the gel. The Lyt-2 subunits (34,000 and 38,000 $M_r$) were also very basic. Under nonreducing conditions, the 65,000–75,000 $M_r$ material clearly separated into two species, with the higher molecular weight species migrating with a more acidic charge (Fig. 5). Similarly, the 140,000 and 200,000 $M_r$ species showed a more acidic charge than any of the three individual subunits. This result may be attributed to (*a*) the macromolecule, because it is composed of disulfide-bonded subunits, may retain some of its conformation under nonreducing conditions that mask some of the basic charge of its subunits; or (*b*) yet another subunit exists as part of the macromolecule that has not been identified because it does not label with $^{125}$I-lactoperoxidase on viable cells; or

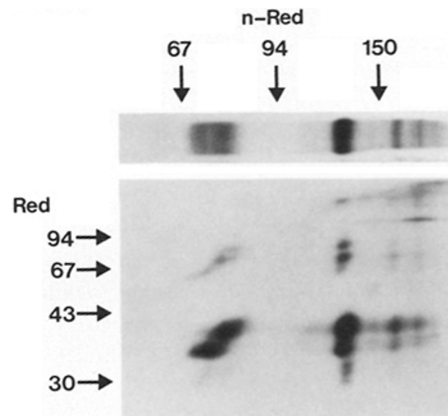


FIG. 4. Two-dimensional gel electrophoresis of the Lyt-2,Lyt-3 macromolecule. A detergent lysate from BALB/cNHz surface $^{125}$I-labeled thymocytes was immunoprecipitated with anti-Lyt-2 (53-6.7) and electrophoresed under nonreducing gel conditions in the first dimension and under reducing gel conditions in the second dimension. The first dimension tube gels were 10% DATD-acrylamide and the second dimension slab gels were 12.5% bisacrylamide-acrylamide. The top of the figure shows a gel track of an anti-Lyt-2 immunoprecipitate run on a 10% DATD-acrylamide gel under nonreducing conditions.

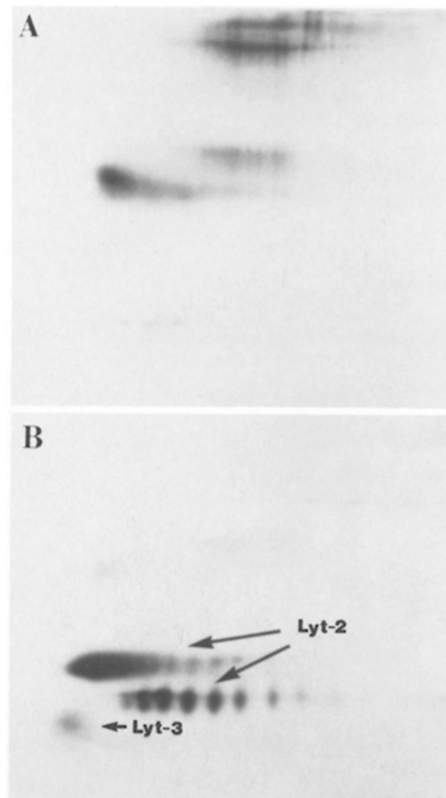


FIG. 5. Two-dimensional gel electrophoresis of the Lyt-2, Lyt-3 macromolecule. A detergent lysate from BALB/cNHz surface $^{125}$I-labeled thymocytes was immunoprecipitated with anti-Lyt-2 (53-6.7). The first dimension was a charge separation with the acidic side on the right and the basic side on the left. The second dimension was 10% SDS-polyacrylamide slab gels from the top to the bottom. A, nonreducing; B, reducing.

(*c*) the larger multimeric forms have more carbohydrate and are more acidic due to additional sialic acid residues.

Immunoprecipitation with anti-Lyt-2 from $^{125}$I-lactoperoxidase-labeled lymph node cells showed some differences in the structure of the macromolecule compared with thymocytes. Under reducing conditions on SDS gels, the 30,000 $M_r$ species that by analogy with thymocytes corresponds to the Lyt-3 subunit was present. However, instead of the 34,000 and 38,000 $M_r$ species seen on thymocytes, only a protein of 38,000 $M_r$ was seen on lymph node cells. Although this latter 38,000 $M_r$ species migrated as a tight double band, the 34,000 $M_r$ species seen on thymoctyes was not present on lymph node cells (results not shown). These results suggest that the Lyt-2 subunit is processed differently in thymus vs. lymph node.

*The Lyt-2,Lyt-3 Macromolecule Does Not Show Allelic Exclusion.* Because of the close genetic association of Lyt-2 and Lyt-3 genes with $\kappa$ light chain loci and the haplotype exclusion demonstrated by $\kappa$ light chains, it was of interest to determine whether the expression of Lyt-2 and Lyt-3 antigens is regulated in a similar way, i.e., by allelic exclusion. Quantitative immunofluorescence of (BALB/cNHz × AKR/J)$F_1$ mice (Lyt-$2^{a/b}$, Lyt-$3^{a/b}$) thymocytes and spleen cells compared with each parental strain

showed that Lyt-2.2 and Lyt-3.1 do not show allelic exclusion on thymocytes or T cells (Table II). In the heterozygote, the antigens are found on the same percentage of cells as the positive parental strain, but are expressed in lower density than on cells from the positive parental strains.

*Trypsin Sensitivity of Lyt-2 and Lyt-3 Antigens on Viable Cells.* Both Lyt-2 and Lyt-3 antigens are efficiently digested from viable cells with low levels of trypsin, whereas Lyt-1 is not removed under these conditions. Fig. 6 shows that Lyt-2 and Lyt-3 antigens on thymocytes exhibit differential sensitivity to trypsin. Lyt-3 antigens are

TABLE II
*Lyt-2 and Lyt-3 Do Not Show Allelic Exclusion*

| Mouse strain (Lyt-2,Lyt-3 phenotype) | FACS analysis* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Thymus | | | | Spleen | | | |
| | Lyt-2.2‡ | | Lyt-3.1§ | | Lyt-2.2‡ | | Lyt-3.1§ | |
| | Mean of positive‖ | % positive | Mean of positive | % positive | Mean of positive | % positive | Mean of positive | % positive |
| AKR/J (Lyt-2.1,3.1) | — | <1 | 380 | 89 | — | <1 | 420 | 13 |
| BALB/cNHz (Lyt-2.2,3.2) | 410 | 85 | — | <1 | 467 | 14 | — | <1 |
| (AKR/J × BALB/cNHz)$F_1$ | 240 | 86 | 210 | 84 | 257 | 13 | 238 | 12 |

* Immunofluorescence staining was analyzed using a fluorescence-activated cell sorter (FACS)-II (Becton Dickinson FACS Division, Sunnyvale, Calif.).

‡ Immunofluorescence staining with anti-Lyt-2.2 (19/178) was done indirectly using a fluorescein-conjugated goat anti-mouse $IgG_2$ second-step antibody.

§ Immunofluorescence staining with anti-Lyt-3.1 [E2(7)] was done indirectly using biotin-conjugated antibody followed by fluorescein-conjugated avidin second-step reagent. The anti-Lyt-3.1 monoclonal antibody [E2(7)] was a gift from Dr. P. Gottlieb, University of Texas, Austin, Tex.

‖ Geometric mean fluorescence of positive cells is expressed in arbitrary linear units.

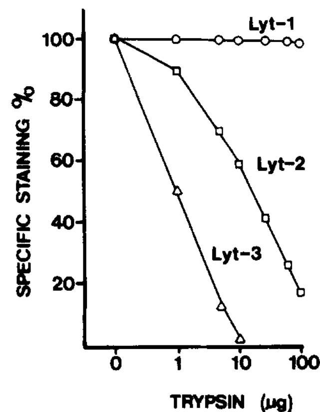


FIG. 6. Trypsin digestion of BALB/cNHz thymocytes. The cells ($4 \times 10^7$/ml) were digested with the indicated amount of trypsin for 10 min at 37°C. The digestion was stopped by the addition of 10 ml of medium containing 15% calf serum. Cells were then stained with fluorescein-conjugated anti-Lyt-1 (53-7.3), fluorescein-conjugated anti-Lyt-2 (53-6.7), and fluorescein-conjugated anti-Lyt-3 (53-5). The percentage of specific staining was calculated by:

$$\frac{\text{(Fluorescence intensity at "X" trypsin)} - \text{(background fluorescence [unstained cells])}}{\text{(Fluorescence intensity at "O" trypsin)} - \text{(background fluorescence [unstained cells])}} \times 100.$$

cleaved more readily than Lyt-2 antigens and can be almost completely removed with >60% of Lyt-2 antigens remaining (Fig. 6). Although the data shown were derived from assays using monoclonal antibodies, similar results were obtained using conventional anti-Lyt alloantisera from several sources (data not shown). The differential trypsin sensitivity of Lyt-2 and Lyt-3 confirms the work of Durda and Gottlieb (13). We also observed differential trypsin sensitivity of Lyt-2 and Lyt-3 on peripheral (spleen and lymph node) T cells (data not shown). Studies with other, arginine-specific, proteases (clostridium protease, *Staphylococcus aureus* V8 protease) indicate that the differential sensitivity of Lyt-2 and Lyt-3 reflects cleavages at arginine-specific sites (data not shown).

*Role of the Lyt-2,Lyt-3 Macromolecule on Cytotoxic T cells.* Cytotoxic T cells generated in vivo against P815 mastocytoma cells were assayed for activity against $^{51}$Cr-labeled P815 in efforts to examine the role of the Lyt-2,Lyt-3 macromolecule in the killing process. We took advantage of the selective trypsin cleavage of the Lyt-3 subunit and the ability to measure residual Lyt-2 and Lyt-3 antigens on the killer population by quantitative fluorescence to examine the relationship of cytotoxic activity to the amount of Lyt-2 and Lyt-3 antigens remaining. The results (Table III) showed that the loss of cytotoxic activity after trypsin treatment did not quantitatively parallel the

TABLE III

*Effect of Trypsin Treatment on Blocking of Cytotoxic Effector Cells with Anti-Lyt-2 and Anti-Lyt-3*

| Trypsin* | Surface Lyt-2‡ | Surface Lyt-3‡ | Blocking antibody§ | | Lytic units‖ (±SEM) | P¶ | Blocking |
|---|---|---|---|---|---|---|---|
| | | | Anti-rat Ig | Monoclonal rat antibody | | | |
| | % | % | | | | | % |
| Experiment 1 | | | | | | | |
| 0 | 100 | 100 | − | — | 60 (±2) | — | |
| | | | + | — | 62 (±4) | | NS** |
| | | | + | Anti-Lyt-1 | 64 (±11) | | NS |
| | | | + | Anti-Lyt-2 | 40 (±1) | <0.005 | 35 |
| | | | + | Anti-Lyt-3 | 21 (±1) | <0.005 | 66 |
| 25 μg | 76 | <5 | − | — | 45 (±2) | | — |
| | | | + | — | 45 (±2) | | NS |
| | | | + | Anti-Lyt-1 | 48 (±3) | | NS |
| | | | + | Anti-Lyt-2 | 22 (±3) | <0.003 | 51 |
| | | | + | Anti-Lyt-3 | 33 (±2) | <0.01 | 27 |
| Experiment 2 | | | | | | | |
| 0 | 100 | 100 | + | — | 27 (±2) | | — |
| | | | + | Anti-Lyt-2 | 14 (±1) | <0.003 | 48 |
| | | | + | Anti-Lyt-3 | 9 (±1) | <0.001 | 67 |
| 25 μg | 84 | <5 | + | — | 21 (±1) | | — |
| | | | + | Anti-Lyt-2 | <5 | <0.001 | >76 |
| | | | + | Anti-Lyt-3 | 13 (±2) | <0.01 | 38 |

* Cytotoxic effector cells at $2 \times 10^7$ cells/0.5 ml were treated with the indicated amount of trypsin for 10 min at 37°C.

‡ Cells were assayed for surface Lyt-2 and Lyt-3 using fluorescein-conjugated 53-6.7 and 53-5 monoclonal antibodies and a FACS-II.

§ Blocking of T cell killing is described in Materials and Methods.

‖ Lytic units were calculated as described in Materials and Methods.

¶ Significance levels calculated with the Student's *t* test.

** Not significant.

loss of Lyt-3 antigens on the cytotoxic T cell population. After almost complete removal of Lyt-3, the cytolytic activity had decreased by ~25%. These assays were repeated several times with similar results. Using a cytotoxic T cell line (L3C5; 20) rather than in vivo-generated cytotoxic T cells, we found the opposite results; the loss of cytolytic activity after trypsin treatment was more rapid than the loss of Lyt-3 antigen (A. Glasebrook, J. A. Ledbetter, and L. A. Herzenberg, unpublished data). In neither case, however, was an exact quantitative correlation seen between cleavage of Lyt-3 and loss of cytolytic activity.

Table III shows that after trypsin treatment, the in vivo-generated cytotoxic effector cells could still kill, and that the blocking of killing by anti-Lyt-3 was significantly reduced, whereas the blocking of killing by anti-Lyt-2 was significantly improved. Although Lyt-3 was not detected by immunofluorescence after trypsin treatment, some blocking of killing by anti-Lyt-3 was still observed (Table III). The blocking assay may be more sensitive than immunofluorescence for detection of low levels of surface Lyt-3. The results imply that killer T cells with very low levels of the Lyt-3 subunit are still functional, and that the activity of the Lyt-2,Lyt-3 molecule relevant to the killing process may not reside in the Lyt-3 subunit.

## Discussion

Lyt-2 and Lyt-3 antigens are controlled by closely linked genes (4). The results of this paper show that Lyt-2 and Lyt-3 antigens are part of the same macromolecule and that they reside on different subunits. This is unlike the situation with some other multimeric molecules such as hemoglobins, immunoglobulins, and H-2 (K or D with $B_2$ microglobulin), in which genes controlling individual subunits are not linked. In other multimeric proteins, such as insulin or the murine leukemia virus envelope gene products, the individual subunits are both coded for by a single gene. This leads to production of a precursor polyprotein, which is processed post-translationally by proteolytic cleavage to generate the separate subunits.

Our findings, together with the previously known close linkage of Lyt-2 and Lyt-3, suggest that the biosynthesis of Lyt-2 and Lyt-3 subunits may proceed through a precursor polyprotein intermediate. Pulse-chase labeling studies could determine whether Lyt-2 and Lyt-3 subunits are synthesized as a polyprotein that is subsequently cleaved.

When analyzed under nonreducing gel conditions, the Lyt-2,Lyt-3 macromolecules are seen in a variety of multimeric forms. Because the antigen densities of Lyt-2 and Lyt-3 measured on thymocytes or T cells are equal (15), the various multimeric forms appear to be hexamers, tetramers, and dimers consisting of equal numbers of Lyt-2 and Lyt-3 subunits. The biochemical analyses (immunoprecipitation and gel electrophoresis) reported here are performed rapidly in the cold and in the presence of protease inhibitors and alkylating reagents, so it is likely that the various multimeric forms of the macromolecule are present on the cell surface rather than being artifactually produced during the isolation procedure.

In addition to the Lyt-2,Lyt-3 multimers, we found small amounts of the monomer Lyt-3 subunit (30,000 $M_r$) in detergent lysates of labeled cells. Some Lyt-$2^+$Lyt-$3^-$ material was present also, in the form of dimers of the 34,000 and 38,000 $M_r$ subunits. These results are qualitatively consistent with the experiments of Durda and Gottlieb (13), who did similar anti-Lyt-2 and anti-Lyt-3 preclearing experiments. These

workers concluded that Lyt-2 and Lyt-3 antigens are separable. We find that the free Lyt-3 and Lyt-2 subunits are minor species in the detergent lysates, whereas the multimeric Lyt-2,Lyt-3 forms account for almost all of the antigenic protein.

The one- and two-dimensional gel patterns that we obtain for the Lyt-2,Lyt-3 macromolecule are very similar to those of Reilly et al. (20). These authors identified two heavily labeled subunits of 35,000 and 30,000 $M_r$ and a faintly labeled subunit of <30,000 $M_r$. Based on their charge and labeling properties, these subunits correspond to the 38,000, 34,000, and 30,000 $M_r$ subunits seen in our experiments. The differences in apparent molecular weight most likely result from differences in the gel systems used.

Our demonstration that Lyt-2 and Lyt-3 antigens reside on separate subunits relied on the retention of antigenic activity after reduction and alkylation in the cold of the NP-40 cell lysate. In these experiments using labeled thymocytes, the 30,000 $M_r$ subunit specifically reacted with anti-Lyt-3, whereas the 34,000 and 38,000 $M_r$ subunits specifically reacted with anti-Lyt-2. It is most likely that these latter two subunits both have Lyt-2 antigenic determinants and arise from a post-translational processing difference involving either carbohydrate or proteolytic cleavage. This interpretation is supported by the observation that only one of the Lyt-2 subunits (38,000 $M_r$) was seen on lymph node T cells. Alternatively, there is the possibility that only one of the two species that coprecipitated with anti-Lyt-2 from thymocytes has the Lyt-2 determinants, whereas the other is tightly associated through strong noncovalent interactions.

Lyt-2 and Lyt-3 antigens on viable cells show differential sensitivity to trypsin cleavage. Lyt-3 antigens can be almost completely removed under conditions in which Lyt-2 antigens are only slightly affected. Our studies of in vivo-generated T killer cells after trypsin treatment suggest that the Lyt-3 subunit is not required for cytolytic activity. This conclusion derives from the observation that trypsin-treated cytotoxic T cells with <5% residual Lyt-3 retain >75% of their cytolytic activity. However, some blocking of killing with anti-Lyt-3 still occurs. Because these experiments were performed with in vivo-generated cytotoxic T cells, the effector cells represent a heterogeneous population of T cell clones. Similar studies with a cytotoxic T cell line (L3C5; 21) showed that the lytic activity was more sensitive to trypsin than was the Lyt-3 subunit. If the Lyt-3 subunit had been directly involved in antigen recognition, we would have seen parallel losses of the antigen and cytolytic activity.

The loci controlling Lyt-2 and Lyt-3 are closely linked to the mouse $\kappa$ chain genes on chromosome 6 (5–7). Although this close linkage may be fortuitous, it is also possible that $V_\kappa$ gene products associate with the Lyt-2,Lyt-3 macromolecule. However, control of expression of these linked genes may be different because Lyt-2 and Lyt-3 are not allelically excluded (on T cells), whereas $V_\kappa$ (on B cells) is allelically excluded. Some evidence for light chain variable region ($V_\lambda$) expression on T cells bearing the Lyt-2,Lyt-3 antigens is available (22), but whether $V_\lambda$ is allelically excluded on these cells is not known.

Comparative structural and functional studies of mouse and human T cell antigens have shown that human cytotoxic/suppressor T cells have a molecule homologous to the mouse Lyt-2,Lyt-3 molecule (23, 24). This human molecule was originally recognized by the heteroantisera used to define the $TH_2^+$ T cell subset (25) and has now been characterized by monoclonal antibodies T5 (26), and SK1 and SK2 (23,

24). The human Lyt-2,Lyt-3 homologue, termed Leu-2, parallels the mouse antigen in its selective expression on a functional subset of T cells and its structure as a multimeric macromolecule composed of individual disulfide-bonded subunits (23). Furthermore, the anti-Leu-2 monoclonal antibodies (SK1 and SK2) parallel the anti-Lyt-2 and anti-Lyt-3 antibodies in the blocking of killing by human cytotoxic T cells in the absence of complement (24).

The maintenance of homologous molecules on functionally distinct T cell subpopulations in two evolutionarily distant species suggests that the Lyt and Leu antigens perform essential functions for the cells on which they are found. The data so far, including (*a*) lack of allelic exclusion, and (*b*) nonparallel losses of the Lyt-3 subunit with T cell cytolytic activity after trypsin treatment, suggest that this role is something other than that of a direct antigen receptor.

## Summary

Lyt-2 and Lyt-3 antigens are carried on separate disulfide-bonded subunits of the same cell surface macromolecules. These are present on thymocytes in a variety of multimeric forms consisting of disulfide-bonded dimers, tetramers, and hexamers of pairwise combinations of three subunits (30,000, 34,000, and 38,000 $M_r$). From reduced and alkylated Nonidet-P40 thymus extracts, a monoclonal anti-Lyt-3 precipitates only the 30,000 $M_r$ subunit, whereas monoclonal anti-Lyt-2 antibody precipitates both the 38,000 and 34,000 $M_r$ subunits, but not the 30,000 $M_r$ subunit. Almost all of the Lyt-2 and Lyt-3 subunits on the cell are covalently linked by disulfide bonds. However, small amounts of the free Lyt-3 subunit was seen in some experiments. Similarly, small amounts of Lyt-$2^+3^-$ material, consisting of dimers of the 38,000 and 34,000 $M_r$ subunits were identified. Each of the three subunits migrated with a basic charge (pI > 8) on two-dimensional gels.

Cytotoxic effector cells that are blocked by anti-Lyt-2 and anti-Lyt-3 can be treated with trypsin and other arginine-specific proteases to remove these antigens. At low concentrations of these proteases, Lyt-3 antigens are selectively removed. After selective removal of Lyt-3 antigens, cytotoxic effector cells are still active and blocking of activity by anti-Lyt-3 is significantly reduced, whereas blocking of activity by anti-Lyt-2 is significantly increased. Neither Lyt-2 nor Lyt-3 is allelically excluded on thymocytes or T cells. These results suggest that the Lyt-2,Lyt-3 macromolecules are associated with but do not serve as the T cell antigen receptor.

The authors thank Dr. P. Gottlieb (University of Texas, Austin, Tex.), who generously provided the anti-Lyt-3.1 monoclonal antibody, and Dr. U. Hämmerling (Memorial Sloan-Kettering Cancer Center, New York), who generously provided the anti-Lyt-2.2 monoclonal antibody. We also thank Ms. Marcia A. Blackman for expert technical assistance and Ms. J. Anderson for preparation of the manuscript.

*Received for publication 24 November 1980 and in revised form 10 March 1981.*

## References

1. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T cell subclasses in a differentiative process independent of antigen. *J. Exp. Med.* **141:**1376.

2. Cantor, H., and E. A. Boyse. 1977. Lymphocytes as models for the study of mammalian cellular differentiation. *Immunol. Rev.* **33:**105.
3. Herzenberg, L. A., K. Okumura, H. Cantor, V. L. Sato, F. W. Shen, E. A. Boyse, and L. A. Herzenberg. 1976. T cell regulation of antibody responses: demonstration of allotype specific helper T cells and their specific removal by suppressor T cells. *J. Exp. Med.* **144:**330.
4. Itakura, K., J. J. Hutton, E. A. Boyse, and L. J. Old. 1972. Genetic linkage relationships of loci specifying differentiation alloantigens in the mouse. *Transplantation.* **13:**239.
5. Gottlieb, P.D. 1974. Genetic correlation of a mouse light chain variable region marker with a thymocyte surface antigen. *J. Exp. Med.* **140:**1432.
6. Claflin, J. L., B. A. Taylor, and M. Cherry. 1978. Linkage in mice of genes controlling an immunoglobulin kappa-chain marker and the surface alloantigen Ly-3 on T lymphocytes. *Immunogenetics.* **6:**379.
7. Gibsen, D. M., B. A. Taylor, and M. Cherry. 1978. Evidence for close linkage of a mouse light chain marker with the Ly-2,3 locus. *J. Immunol.* **121:**1585.
8. Nakayama, E., H. Shiku, E. Stockert, H. F. Oettgen, and L. J. Old. 1979. Cytotoxic T cells; Lyt phenotype and blocking of killing activity by Lyt antisera. *Proc. Natl. Acad. Sci. U. S. A.* **76:**1977.
9. Shinohara, N., and D. H. Sachs. 1979. Mouse alloantibodies capable of blocking cytotoxic T cell function. I. Relationship between the antigen reactive with blocking antibodies and the Lyt-2 locus. *J. Exp. Med.* **150:**432.
10. Nakayama, E., W. Dippold, H. Shiku, H. F. Oettgen, and L. J. Old. 1980. Alloantigen-induced T-cell proliferation: Lyt phenotype of responding cells and blocking of proliferation by Lyt antisera. *Proc. Natl. Acad. Sci. U. S. A.* **77:**2890.
11. Hollander, N., E. Pillemer, and I. L. Weissman. 1980. Blocking effect of Lyt-2 antibodies on T cell functions. *J. Exp. Med.* **152:**674.
12. Durda, P. J., and P. D. Gottlieb. 1976. The Ly-3 antigens on mouse thymocytes: immune precipitation and molecular weight characterization. *J. Exp. Med.* **144:**476.
13. Durda, P. J., and P. D. Gottlieb. 1978. Sequential precipitation of mouse thymocyte extracts with anti-Lyt-2 and anti-Lyt-3 sera. I. Lyt-2.1 and Lyt-3.1 antigenic determinants reside on separable molecular species. *J. Immunol.* **121:**983.
14. Ledbetter, J. A., and L. A. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* **47:**63.
15. Ledbetter, J. A., R. V. Rouse, H. S. Micklem, and L. A. Herzenberg. 1980. T cell subsets defined by expression of Lyt-1,2,3 and Thy-1 antigens. Two parameter immunofluorescence and cytotoxic analysis with monoclonal antibodies modifies current views. *J. Exp. Med.* **152:** 280.
16. Laemmli, U. K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227:**680.
17. Baumann, G., and A. Chrambach. 1976. A highly crosslinked, transparent polyacrylamide gel with improved mechanical stability for use in isoelectric focusing and isotachophoresis. *Anal. Biochem.* **70:**32.
18. O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell.* **12:**1133.
19. Seaman, W. E., M. A. Blackman, T. D. Gindhart, J. R. Roubinian, J. M. Loeb, and N. Talal. 1978. $\beta$-Estradiol reduces natural killer cells in mice. *J. Immunol.* **121:**2193.
20. Reilly, E. B., K. Auditore-Hargreaves, U. Hämmerling, and P. D. Gottlieb. 1980. Lyt-2 and Lyt-3 alloantigens: precipitation with monoclonal and conventional antibodies and analysis on one and two-dimensional polyacrylamide gels. *J. Immunol.* **125:**2245.
21. Glasebrook, A. L., and F. W. Fitch. 1979. T-cell lines which cooperate in generation of specific cytolytic activity. *Nature (Lond.).* **278:**171.
22. Lonai, P., Y. Ben-Neriah, L. Steinman, and D. Givol. 1978. Selective participation of

immunoglobulin V region and major histocompatibility complex products in antigen binding by T cells. *Eur. J. Immunol.* **8:**827.

23. Ledbetter, J. A., R. L. Evans, M. Lipinski, C. Cunningham-Rundles, R. A. Good, and L. A. Herzenberg. 1981. Evolutionary conservation of surface molecules that distinguish T lymphocyte helper/inducer and cytotoxic/suppressor subpopulations in mouse and man. *J. Exp. Med.* **153:**310.
24. Evans, R. L., D. W. Wall, C. D. Platsoucas, F. P. Siegal, S. M. Fikrig, C. M. Testa, and R. A. Good. 1981.Thymus-dependent membrane antigens in man: inhibition of cell-mediated lympholysis by monoclonal antibodies to the $T_{H2}$ antigen. *Proc. Natl. Acad. Sci. U. S. A.* **78:** 544.
25. Evans, R. L., J. M. Breard, H. Lazarus, A. C. Penta, and S. F. Schlossman. 1978. Two functionally distinct subpopulations of human T cells that collaborate in the generation of cytotoxic cells responsible for cell-mediated lympholysis. *J. Immunol.* **129:**1423.
26. Reinherz, E. L., P. C. Kung, G. Goldstein, and S. F. Schlossman. 1980. A monoclonal antibody reactive with the human cytotoxic/suppressor T cell subset previously defined by a heteroantiserum termed $TH_2$. *J. Immunol.* **124:**1301.