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Immunology

Mouse Lyt-2 antigen: Evidence for two heterodimers with a common subunit

(T lymphocytes/differentiation antigens/T-cell receptors)

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ABSTRACT **Immunoprecipitation of [^{35}S]methionine-labeled extracts of BALB/c thymocytes with mouse Lyt-2.2 monoclonal antibody yielded three components with subunit M_rs of 37,000, 32,000, and 28,000 on NaDodSO$_4$/polyacrylamide gel in the presence of a reducing agent. Two-dimensional polyacrylamide gel analysis revealed that, in the absence of a reducing agent, the three polypeptide chains exist in the form of two heterodimers, each consisting of one molecule of a M_r 28,000 subunit covalently associated through disulfide bonds with either one M_r 37,000 subunit or one M_r 32,000 subunit. These two molecular structures are present in about equimolar ratios in the immunoprecipitate. Immunoautoradiographic analysis after electrophoretic transfer of proteins from a NaDodSO$_4$/polyacrylamide gel to a nitrocellulose membrane indicated that the Lyt-2.2 determinant detected by the monoclonal antibody resides on the M_r 28,000 component, the common subunit of both heterodimeric structures. Lyt-2 precipitated from extracts of different T-cell growth factor-dependent cloned T-cell lines also showed similar structures, although the exact apparent M_rs of the respective components varied somewhat. The structure of the Lyt-2 antigen is of importance, particularly in the light of recent suggestions that it may be involved in the construction of one class of T-cell receptors.**

Three systems of surface antigens, Lyt-1, Lyt-2, and Lyt-3, characterize cells of thymic derivation in the mouse (1, 2). As no other normal cell type has been found to express these components, Lyt antigens have been particularly useful as T-cell markers. Lyt-1 antigens, coded for by a locus with two alleles on chromosome 19 (3), appear to be expressed on all classes of T cells. Lyt-2,3 antigens, on the other hand, clearly distinguish subclasses of T cells (4–8). Cytotoxic and suppressor T cells express Lyt-2 and Lyt-3, whereas helper T cells generally lack these antigens. Lyt-2 and Lyt-3 are coded for by a single gene or closely linked genes on chromosome 6 (9); two allelic forms of both antigens are known.

Considerable interest in Lyt-2,3 antigens has been generated by the recent finding that conventional and monoclonal antibodies to these determinants in the absence of added complement can block T-cell cytotoxicity (10–15). Lyt-2,3 blocking is particularly striking, as antibodies to a range of other antigens on the surface of killer T cells, including Lyt-1 and H-2, did not block. Blocking is not restricted to T-cell reactions involving lysis; alloantigen-induced T-cell proliferation also is inhibited in an allele-specific fashion by antibody to Lyt-2,3 antigens (16, 17). Among the several possibilities considered to account for blocking is steric hindrance of antigen recognition or binding by T cells. This would be the case if molecules bearing Lyt-2,3 determinants were in close spacial proximity to antigen receptors on T cells, either on unrelated adjacent molecular structures or as an integral component of the receptor. The finding that *Lyt-2,3* genes are tightly linked to structural genes for κ light chains on chromosome 6 (18–20) adds support to the speculation that Lyt-2,3 components may be involved in the construction of one class of T-cell receptors in which κ sequences and Lyt-2,3 chains would comprise subunits of a functional recognition unit.

The biochemical analysis of Lyt-2,3 antigen is at too early a stage to provide evidence for or against this role for products of the *Lyt-2,3* locus. Characterization by Durda and Gottlieb (21, 22) with conventional Lyt-2 and Lyt-3 antisera indicated that the determinants for these antigens reside on separable species with M_rs in the range of 35,000. Analysis with monoclonal Lyt reagents has shown that the native molecular forms of Lyt-2,3 molecules exist as dimers having a M_r in the 65,000 range, consisting predominantly of subunits at M_r 30,000–35,000 (23, 24). A component of M_r <30,000 was detected by Reilly *et al.* (24), but this was poorly labeled, and its significance could not be established. In recent work by Ledbetter *et al.* (25), three components were identified by Lyt-2 or Lyt-3 monoclonal antibodies—M_rs 38,000, 34,000, and 30,000. The results of reduction and alkylation experiments indicated that the Lyt-3 determinant is carried on the M_r 30,000 subunit and the Lyt-2.2 determinant is on the M_r 34,000 or 38,000 subunits, or both. These previous studies aimed at defining Lyt-2,3 components have been carried out with cells labeled with ^{125}I by the lactoperoxidase method. In the present study, we characterize the Lyt-2.2 antigen by using [^{35}S]methionine-labeled thymocytes and cloned T-cell lines as targets.

MATERIALS AND METHODS

Mice. Derivation of the B6 Lyt congenic stocks, B6-Lyt-1.1 and B6-Lyt-2.1-Lyt-3.1 has been described (4).

Antisera. The mouse Lyt-2.2 monoclonal antibody (γ2aκ) was a product of hybridoma 19/178 originally derived by U. Hämmerling of the Memorial Sloan–Kettering Cancer Center and used in the form of serum from hybridoma-bearing *nu/nu* mice.

Cells. Thymocytes from 10-wk-old mice and cloned T-cell growth factor-dependent cytotoxic T-cell lines of C57BL/6 and (BALB/c × C57BL/6)F$_1$ origin (26) were labeled for 4 hr in minimum essential medium containing 10% fetal calf serum and 100 μCi/ml (1 Ci = 3.7 × 10^{10} becquerels) of [^{35}S]methionine (>1,000 Ci/mmol). After washing in Tris-buffered saline, the cells were lysed with 50 mM Tris·HCl, pH 7.5/150 mM NaCl/1% Triton X-100/1% sodium deoxycholate/0.1% NaDodSO$_4$. The lysates were clarified by centrifugation at 100,000 × g for 30 min at 4°C.

Immunoprecipitation. Indirect immunoprecipitation with formalin-fixed *Staphylococcus aureus* (Cowan 1 strain) was performed as described (27).

Polyacrylamide Gel Electrophoresis. Immunoprecipitates were dissolved in 62.5 mM Tris·HCl, pH 6.8/2% NaDodSO$_4$/5% 2-mercaptoethanol/10% glycerol. For nonreducing condi-

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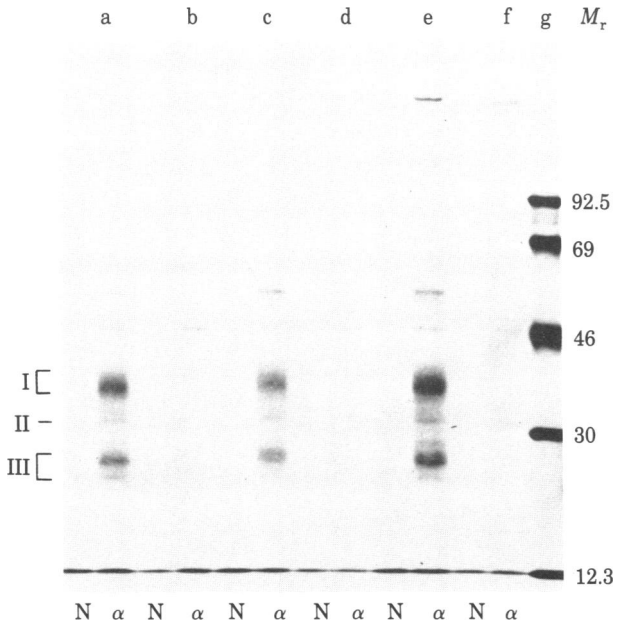

FIG. 1. One-dimensional gel electrophoresis under reducing conditions of immunoprecipitates obtained from thymocytes of different mouse strains. Lanes: a, BALB/c; b, AKR; c, B6-Lyt-1.1; d, B6-Lyt-2.1-Lyt-3.1; e, B6-TL$^+$; f, DBA/2; g, M_r markers. Extracts of thymocytes labeled in culture with [^{35}S]methionine were immunoprecipitated with either normal mouse serum (lanes N) or Lyt-2.2 monoclonal antibody (lanes α). The immunoprecipitates were electrophoresed on a 10% polyacrylamide/NaDodSO$_4$ gel under reducing conditions. M_r markers were phosphorylase *b* (92,500), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and cytochrome *c* (12,300); M_rs are shown $\times 10^{-3}$.

tions, the 2-mercaptoethanol was omitted. The analysis was carried out by electrophoresis on either 10% or 12.5% polyacrylamide gels (28). In two-dimensional analysis, the first dimension was performed under nonreducing conditions in disc gels, and the second dimension was carried out under reducing conditions in slab gels. After electrophoresis, the gels were fluorographed, dried, and exposed to Kodak XAR-5 films at −70°C.

Electrophoretic Transfer of Proteins. Proteins resolved on a NaDodSO$_4$/polyacrylamide gel were electrophoretically transferred to a nitrocellulose membrane, essentially as described by Towbin *et al.* (29). Antibody binding was performed at 4°C for 16 hr with a 1:50 dilution of antiserum. The nitrocellulose membrane was then washed in 25 mM Tris·HCl, pH 7.5/150 mM NaCl/2 mM EDTA/0.1% Triton X-100. Incubation with ^{125}I-labeled protein A (1×10^6 cpm/ml) was at 24°C for 2 hr, followed by extensive washing of the membrane with the same buffer.

RESULTS

Specificity of Reactions with Lyt-2.2 Monoclonal Antibody. Extracts of [^{35}S]methionine-labeled BALB/c thymocytes were immunoprecipitated with mouse Lyt-2.2 monoclonal antibody. Three components, designated I ($M_r \approx 37{,}000$), II ($M_r \approx 32{,}000$) and III ($M_r \approx 28{,}000$), could be resolved by electrophoresis on a 10% polyacrylamide/NaDodSO$_4$ gel in the presence of a reducing agent (Fig. 1). The relative intensities of the various components were not altered when the labeling time was extended from 2 to 4 hr or when the 2-hr labeling was followed by a 4-hr chase in the absence of label. Component I appeared diffuse, suggesting the heterogeneity characteristic of certain glycoproteins in which the composition of the carbohydrate moieties is not constant. Whereas component II seemed to be composed of a more discrete band, component III was made up of several species. A minor protein with a subunit M_r of 55,000 was also detected in amounts varying from experiment to experiment. Conventional Lyt-2.2 antiserum precipitated the same set of components from BALB/c thymocytes. None of the components were precipitated by Thy-1.2, TL, H-2^d, or H-2^b antisera.

The specificity of the Lyt-2.2 monoclonal antibody was further established by analyzing thymocytes from several other strains expressing either the *Lyt-2.2* or the *Lyt-2.1* allele. The complex of the M_r 37,000, 32,000, and 28,000 components was detected with thymocytes expressing the *Lyt-2.2* allele (BALB/c, B6-Lyt-1.1, B6-TL$^+$) but not with thymocytes expressing the alternative allele (AKR, B6-Lyt-2.1-Lyt-3.1, DBA/2) (Fig. 1).

Structure of the Lyt-2.2 Antigen. To determine whether the various components precipitated by Lyt-2.2 antibody are subunit structures of a complexed antigen joined through disulfide bonds, we resolved the immunoprecipitates on a 12.5% polyacrylamide/NaDodSO$_4$ gel under nonreducing conditions. Whereas treatment of immunoprecipitates with 2-mercaptoethanol yielded three components with M_rs of 37,000, 32,000, and 28,000 (Fig. 2*A*), analysis of immunoprecipitates in the absence of sulfhydryl reagents showed major components migrating with M_rs between 55,000 and 75,000 as well as larger components (Fig. 2*B*). The fact that none of the M_r 37,000, 32,000, or 28,000 components was detected under nonreducing conditions suggested that all three must be present as higher M_r structures held together through disulfide bridges. It is worth noting that the apparent M_rs of the three subunit components changed somewhat with the change in polyacrylamide gel concentration (see Fig. 1 and Fig. 2*A*).

To determine the exact subunit composition of the M_r 55,000–75,000 complexes, the immunoprecipitates were subjected to two-dimensional polyacrylamide gel analysis. The im-

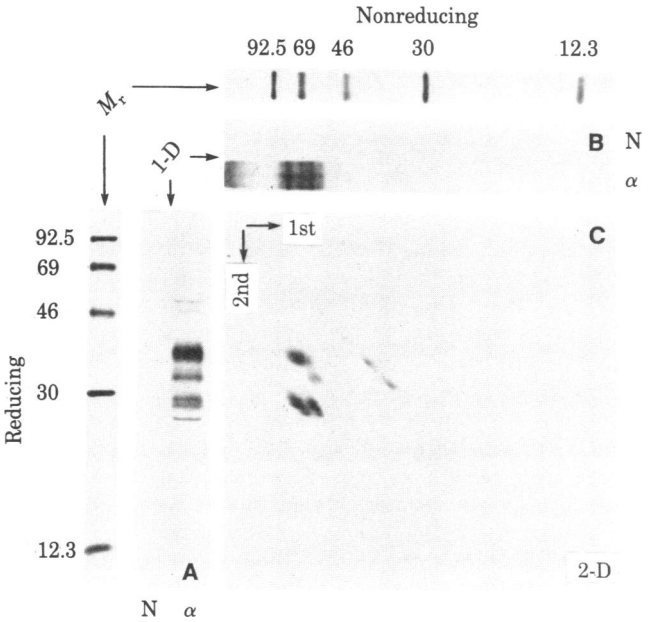

FIG. 2. One- and two-dimensional gel electrophoresis of Lyt-2.2 immunoprecipitates under either reducing or nonreducing conditions. Extracts of BALB/c thymocytes labeled with [^{35}S]methionine were immunoprecipitated with either normal mouse serum (N) or Lyt-2.2 monoclonal antibody (α). For one-dimensional (1–D) analysis, the immunoprecipitates were analyzed on 12.5% polyacrylamide/NaDodSO$_4$ gels under either reducing (*A*) or nonreducing (*B*) conditions. M_r markers were the same as those in Fig. 1; M_rs are shown $\times 10^{-3}$. For two-dimensional (2-D) analysis, the immunoprecipitates obtained with the Lyt-2.2 monoclonal antibody were first fractionated on a 12.5% disc gel under nonreducing conditions and subsequently fractionated on a 12.5% slab gel under reducing conditions (*C*).

mune complexes were first fractionated under nonreducing conditions in the first dimension and then analyzed under reducing conditions in the second dimension (Fig. 2*C*). The results are consistent with the existence of two types of complexes, each containing one M_r 28,000 molecule covalently associated with either one M_r 37,000 or one M_r 32,000 molecule. Clearly excluded is the existence of homodimers of the M_r 37,000, M_r 32,000, or M_r 28,000 molecules or the existence of heterodimers between the M_r 37,000 and M_r 32,000 components. The two-dimensional analysis further amplified the heterogeneity of the three kinds of subunits, particularly the M_r 28,000 species. Such heterogeneity would explain the broad distribution of the nonreduced complexes. In addition, the minor M_r 55,000 component does not appear to exist as part of these disulfide-linked structures.

Because the two types of complexes share a common M_r 28,000 subunit, it is possible to quantitate the abundance of the two molecular forms despite their possible differences in methionine content. The relative intensity of the M_r 28,000 component associated with the two molecular forms suggest that the $[(M_r\ 37{,}000)_1 + (M_r\ 28{,}000)_1]$ complex and the $[(M_r\ 32{,}000)_1 + (M_r\ 28{,}000)_1]$ complex are present in about equimolar ratios in the immunoprecipitate, and that the M_r 32,000 subunit contains relatively fewer methionines than the M_r 37,000 subunit.

Subunit Localization of the Lyt-2.2 Determinant. Immunoautoradiographic analysis after electrophoretic transfer of proteins from $NaDodSO_4$/polyacrylamide gels to nitrocellulose membranes was used to determine which of the subunits contain the Lyt-2.2 determinant detected by the monoclonal antibody. Total protein from an extract of unlabeled BALB/c thymocytes first was fractionated on a 12.5% polyacrylamide/$NaDodSO_4$ gel under reducing conditions and then was transferred electrophoretically to a sheet of nitrocellulose membrane. The latter then was incubated successively with antibody and ^{125}I-labeled protein A.

The results showed that only one component, with a M_r identical to that of the M_r 28,000 subunit, was detected with the Lyt-2.2 monoclonal antibody with this procedure (Fig. 3). This component was not detected by normal mouse serum or by antibodies to unrelated antigens (e.g., p53, H-2^d, and H-2^b). It appears, therefore, that the M_r 28,000 subunit retains its ability to be recognized by Lyt-2.2 monoclonal antibody even when dissociated from the M_r 37,000 or M_r 32,000 subunit by reduction. However, our inability to detect reactivity with the M_r 37,000 and M_r 32,000 subunits does not exclude the possibility that either polypeptide chain may exhibit reactivity in their native conformation under nonreducing and nondenaturing conditions.

Analysis of Cytotoxic T-Cell Lines. In an attempt to understand the basis for the complexity of the Lyt-2 antigen, we analyzed three T-cell growth factor-dependent cloned T-cell lines. $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions of Lyt-2.2 immunoprecipitates from extracts of these cytotoxic T-cell lines also showed the presence of three components (Fig. 4). Component I migrated with a broad distribution, suggesting even more heterogeneity than that seen with thymocytes. In addition, the apparent M_r of this component differed somewhat between the individual cell lines; the component from two subclones of the RLδ1 cytotoxic line (CTLL-R1 and CTLL-R5) migrated slightly behind that from CTLL-A2. Component II, apparently composed of a single protein band on the gel, had the same migration as component II from thymocytes. Comparisons of the T-cell lines and thymocytes showed considerable variation in component III. In the case of the CTLL-R1 and CTLL-R5 clones, component III seemed to be composed of three discrete M_r species, and this pattern differed from that seen with either CTLL-A2 or thymocytes. The

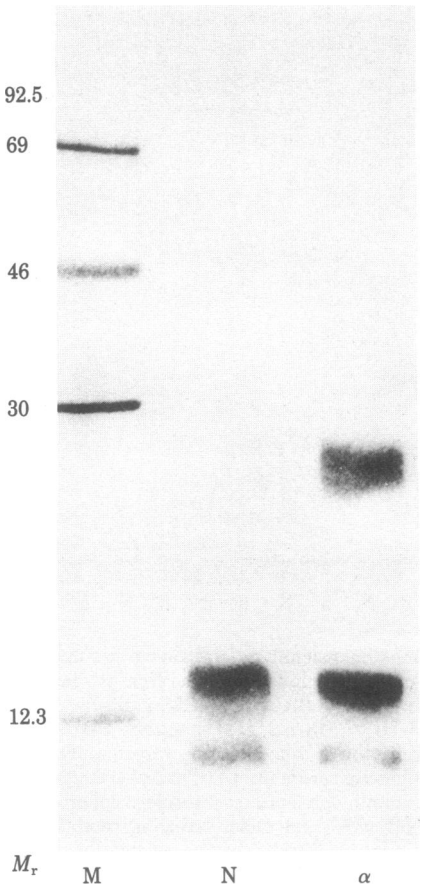

FIG. 3. Immunoautoradiographic detection of the Lyt-2.2 determinant after electrophoretic transfer of proteins from $NaDodSO_4$/polyacrylamide gels to nitrocellulose membranes. Total protein from BALB/c thymocytes was fractionated on a 12.5% polyacrylamide/$NaDodSO_4$ gel under reducing conditions and then transferred electrophoretically to a nitrocellulose membrane. After incubation with either normal mouse serum (lane N) or Lyt-2.2 monoclonal antibody (lane α), the membrane was treated with ^{125}I-labeled protein A and subjected to autoradiography. M_r markers (lane M) were the same as those in Fig. 1; M_rs are shown $\times 10^{-3}$. The two components migrating in the vicinity of the M_r 12,300 marker and detected by both normal serum and antibody are presumably protein A-binding proteins.

minor M_r 55,000 component seen in thymocytes did not seem to accumulate in any of the T-cell lines.

Two-dimensional gel electrophoresis of Lyt-2.2 immunoprecipitates from the T-cell lines showed that the three components existed as $[(M_r\ 37{,}000)_1 + (M_r\ 28{,}000)_1]$ and $[(M_r\ 32{,}000)_1 + (M_r\ 28{,}000)_1]$ heterodimers, the same molecular structures found in Lyt-2.2 immunoprecipitates of thymocytes.

DISCUSSION

Three polypeptide species of M_rs 37,000, 32,000, and 28,000 were identified in extracts of [^{35}S]methionine-labeled thymocytes or cloned T-cell lines by immunoprecipitation with Lyt-2.2 monoclonal antibody and $NaDodSO_4$/polyacrylamide gel electrophoresis of the precipitate under reducing conditions. Analysis under nonreducing conditions revealed higher M_r species in the immunoprecipitate, with M_rs ranging from 55,000 to 75,000. By using two-dimensional polyacrylamide gel electrophoresis in which immunoprecipitates were first fractionated under nonreducing conditions and then analyzed under reducing conditions, the higher M_r species could be clearly resolved into two separate heterodimers of $[M_r\ 37{,}000)_1 + (M_r\ 28{,}000)_1]$ and $[(M_r\ 32{,}000)_1 + (M_r\ 28{,}000)_1]$. The larger subunits in these heterodimers were clearly identified in past studies of Lyt-2 by

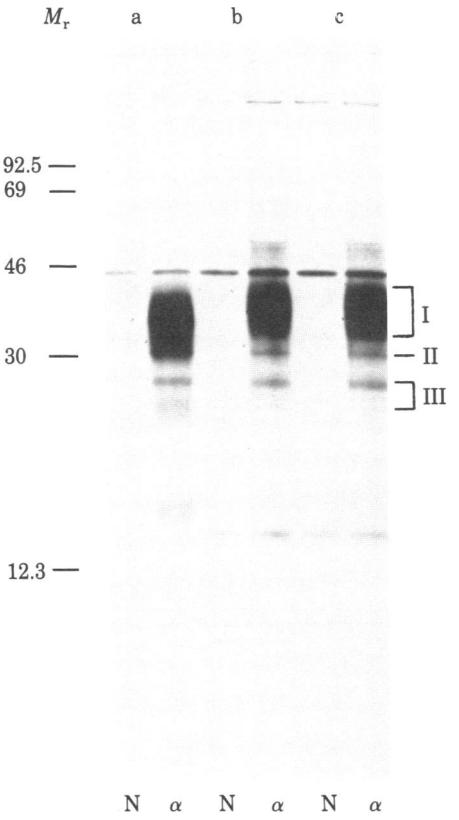

FIG. 4. One-dimensional gel electrophoresis under reducing conditions of immunoprecipitates obtained from extracts of different cloned T-cell lines. Lanes: a, CTLL-A2; b, CTLL-R1; c, CTLL-R5. Extracts of cells labeled with [^{35}S]methionine were immunoprecipitated with either normal mouse serum (lanes N) or Lyt-2.2 monoclonal antibody (lanes α). The immunoprecipitates were electrophoresed on a 12.5% polyacrylamide/NaDodSO$_4$ gel under reducing conditions. M_r markers were the same as those in Fig. 1; M_rs are shown $\times 10^{-3}$. The M_r 43,000 component nonspecifically precipitated by both normal serum and antibody is actin.

using ^{125}I-surface-labeled thymocytes (23, 24). However, the M_r 28,000 component appears to be a poor substrate for labeling by ^{125}I. It was not detected by Ledbetter and Herzenberg in their initial analysis of Lyt-2 by a rat monoclonal antibody (23), and they concluded that the M_r 65,000 component consisted of heterodimers of [$(M_r\ 35{,}000)_1$ + $(M_r\ 30{,}000)_1$]. In the study of Reilly *et al.* (24), a component smaller than M_r 30,000 was detected in some Lyt-2.2 immunoprecipitates, but its presence was quite variable and, therefore, its significance was difficult to evaluate. Their analysis of the M_r 65,000 species indicated complexes containing [$(M_r\ 35{,}000)_2$] and [$(M_r\ 30{,}000)_2$] homodimers, although they could not exclude participation of the M_r <30,000 component, which is probably equivalent to the M_r 28,000 component detected in this study. Because of the better labeling of the M_r 28,000 component with [^{35}S]methionine, it appears that [$(M_r\ 37{,}000)_1$ + $(M_r\ 28{,}000)_1$] and [$(M_r\ 32{,}000)_1$ + $(M_r\ 28{,}000)_1$] heterodimers represent the dominant components precipitated by Lyt-2.2 antibody.

Immunoautoradiographic analysis of protein blots showed that the Lyt-2.2 determinant resides on the M_r 28,000 subunit. This would account for the immunoprecipitation by Lyt-2.2 antibody of two heterodimeric structures in which the M_r 28,000 subunit is an invariant component of both heterodimers. From their analyses of reduced and alkylated extracts of ^{125}I-labeled thymocytes, Ledbetter *et al.* (25) came to a different conclusion with regard to the localization of the Lyt-2.2 determinant. They found that one or both of the larger subunits (M_r 38,000 and M_r 34,000 in their gel systems) reacted with Lyt-2.2 antibody, whereas the smaller subunit (M_r 30,000 in their system) did not. We have repeated such reduction/alkylation experiments with [^{35}S]methionine-labeled extracts but were unable to localize the Lyt-2.2 determinants by this approach because of the apparently nonselective loss of reactivity with all three subunits under conditions of complete reduction and alkylation.

The finding that the Lyt-2.2 determinant resides on the M_r 28,000 subunit suggests that this polypeptide is a product of the *Lyt-2,3* locus. The genetic origin of the other subunits comprising the two Lyt-2 heterodimers is unknown, the possibilities being that they also are coded for by the *Lyt-2,3* locus or by closely linked genes (e.g., κ genes) or by loci elsewhere in the genome. Structural analysis of the Lyt-2 subunits may help to resolve this issue and provide insight into the significance of the close linkage of genes coding for Lyt-2,3 and κ light chains and the meaning of specific blocking of cytotoxic T cells by antibodies directed against Lyt-2,3 antigens.

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