Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 1519–1523, March 1984
Immunology

Isolation and partial characterization of concanavalin A receptors on cloned cytotoxic T lymphocytes

(cloned T cells/cell-surface antigens/lectins/γ-interferon/glycoproteins)

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Contributed by Herman N. Eisen, November 17, 1983

ABSTRACT **A small set of concanavalin A (Con A)-binding glycoproteins was isolated from the surface membrane of cloned cytotoxic T lymphocytes (CTL) and partly identified using monoclonal antibodies. The binding of Con A by these glycoproteins on the CTL surface results in the secretion of γ-interferon and in blocking the effector functions of the cells—namely, antigen-specific and lectin-dependent cytotoxicity. The Con A is evidently bound tightly to some surface structures ("Con A-receptors") that are required for the activation and cytotoxic activity of CTL. To isolate and identify these receptors, antibodies to Con A were used. After Con A was allowed to bind to radiolabeled cloned CTL (labeled with ^{125}I or [^{35}S]methionine or ^{3}H-labeled amino acids), the cells were washed thoroughly, lysed in detergents and anti-Con A antibodies were added to bind to the Con A–receptor complexes. The resulting aggregates were adsorbed with protein A-bearing Staphylococci and the receptors were then specifically released from the pelleted bacteria by α-methyl-D-mannoside and analyzed by polyacrylamide gel electrophoresis under reducing conditions. Eight to nine labeled components were seen by autoradiography and with the aid of monoclonal antibodies to known T-cell surface molecules, four were identified as T200, lymphocyte function-associated antigen (LFA)-1, α- and β-chains, and (on some clones) Lyt-2. Other components with $M_r \simeq$ 160,000, 120,000, 46,000, 42,000, and 23,000 have not been identified. The procedures described here may have general application in the studies of the functional properties of other cell surface molecules.**

Cytotoxic T lymphocytes (CTL) are distinguished by their ability to lyse target cells by reactions that depend on specific recognition of target-cell surface antigens. The antigens are usually either (*i*) foreign class I molecules encoded by the major histocompatibility complex (H2-K, H2-L, H2-D in the mouse) or (*ii*) any of a great variety of other target-cell surface antigens that are recognized in conjunction with class I molecules on the same target cells (1–3). The usual way of identifying the CTL surface molecules that might be involved in recognition, adhesion to, or lysis of target cells has been through inhibition of cytotoxic activity by antibodies to CTL surface antigens (4–7). Because immunogenicity is not always predictable and because the critical molecules on CTL are likely to be glycoproteins, we previously explored the use of lectins, in place of antibodies, as selective inhibitors of CTL activity (8). Several lectins were tested and one, concanavalin A (Con A), turned out to be promising: at low, nonagglutinating concentrations, Con A bound to CTL and blocked their ability to lyse their specific target cells; the effect was carbohydrate specific and could be completely reversed by stripping bound Con A from the CTL with α-methylmannoside (8, 9). It was not clear, however, whether the surface structures that are required for CTL activity actually bind Con A, or whether they are unable to bind it but are simply near neighbors of molecules that do. Some of the studies described here provide evidence that Con A-binding structures (hereafter called Con A receptors) on CTL are themselves involved in reactions with target cells. We have, accordingly, developed procedures based on the use of Con A and anti-Con A antibodies to isolate and characterize these receptors.

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MATERIALS AND METHODS

CTL and Assay for Cytotoxicity. Cloned CTL lines (G4, B10, 1D, 2C) were derived and maintained as described (10, 11). H-2-restricted CTL clone cr 15 (12) was provided by M. Bevan.

Cytotoxic activity was measured in a standard 4-hr ^{51}Cr-release assay (13), using ^{51}Cr-labeled P815 cells (H-2^d) as specific targets.

Antibodies. Monoclonal antibodies (mAb) to T-cell surface antigens were used as unfractionated culture supernatants from hybridoma cell cultures. Hybridoma culture supernatants containing rat mAb to T200 (M 1/9.3, IgG) and rat mAb to lymphocyte function-associated antigen (LFA)-1 (M 7/14, IgG) were provided by T. Springer (6). Culture supernatants containing another rat mAb to T200 (I3/2.3 IgG) was furnished by I. Trowbridge (14). Hybridoma cells 3.155 were a gift from F. Fitch; these cells produce a rat mAb (IgM) to Lyt-2 (15).

Conventional mouse anti-Lyt-2 alloantiserum was prepared in this laboratory (16). Rabbit antiserum to Con A and purified rabbit anti-Con A antibodies were obtained from Vector Laboratories (Burlington, CA) or were prepared by immunizing rabbits with Con A and affinity purifying the anti-Con A antibodies on a Con A-Sepharose column in the presence of α-methylmannoside.

Concanavalin A. This lectin and fluorescein isothiocyanate (FITC)-labeled and rhodamine isothiocyanate (RITC)-labeled Con A was purchased from Vector Laboratories.

Fluorescence-Activated Cell Sorter Analyses. Cells with adsorbed FITC-labeled antibodies or RITC-labeled Con A were analyzed in a flow cytometer (Cytofluorograph System GOH, Ortho Diagnostic), usually with 30,000 cells per run. For FITC-labeled and RITC-labeled ligands, the excitation source (argon ion laser) was set at 500 mW of output at 488 nm and 514 nm, respectively; the detectors were set at 520–540 nm (for FITC) and >610 nm (for RITC). For indirect

Abbreviations: Con A, concanavalin A; CTL, cytotoxic T lymphocytes; Con A–CTL, CTL with tightly bound Con A; FITC, fluorescein isothiocyanate; γ-IFN, γ-interferon; LFA, lymphocyte function-associated antigen; mAb, monoclonal antibody; RITC, rhodamine isothiocyanate.

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immunofluorescence, FITC-labeled rabbit anti-mouse Ig and FITC-labeled goat anti-rat Ig (both from Cappel Laboratories, Cochranville, PA) were used as second antibodies.

Radiolabeling of Lymphocytes. Cultured cells were centrifuged through Ficoll-Hypaque on a discontinuous 17.5%–35% bovine serum albumin gradient, and the recovered cells (>95% viable) were iodinated with ^{125}I and lactoperoxidase (13). To label cell surface components metabolically, cloned CTL (G4 and 1D) were incubated with [^{35}S]methionine or a mixture of ^{3}H-labeled amino acids (New England Nuclear) in amino acid-depleted RPMI 1640 ("Select Amino Acid Kit", GIBCO), containing 10% dialyzed fetal calf serum. After 14–20 hr at 37°C, dead cells were removed by centrifugation through Ficoll-Paque.

Isolation of Con A Receptors. Briefly, the procedures used to isolate these structures involved incubating the radiolabeled cells with Con A, washing away unbound Con A, lysing the resulting cells, and specifically immunoprecipitating the solubilized Con A–receptor complexes with anti-Con A antibodies and protein A-bearing *Staphylococcus aureus* (Cowan I) ("Staph A").

The CTL were first surface-labeled with ^{125}I or metabolically labeled with [^{35}S]methionine, and then the washed cells were incubated for 45 min at 37°C at 1×10^6 cells per ml in serum-free RPMI 1640 containing 2 mg of bovine serum albumin per ml (0.2% albumin/RPMI 1640) and 2.5 μg of Con A per ml (9). After removing unbound Con A by three cycles of centrifugation in ice-cold 0.2% albumin/RPMI 1640, the resulting cells (called Con A–CTL) were lysed by suspending them at 2×10^7 cells per ml in Con A extraction buffer: 0.5% Nonidet P-40/150 mM NaCl/50 mM Tris·HCl, pH 7.5/2 mM phenylmethylsulfonyl fluoride/1 mM $CaCl_2$/1 mM $MgCl_2$/0.01% albumin/0.02% NaN_3. The suspension was mixed vigorously for 5–10 sec in a Vortex mixer and then incubated at 0°C for 15 min with intermittent mixing. Nuclei and debris were removed by centrifugation (Eppendorf, Model 3200) in the cold at $8000 \times g$ for 2–3 min. The supernatants (Con A–CTL extracts) were depleted of nonspecifically binding components by twice incubating them with normal mouse or rabbit antiserum (25 μl of antiserum per ml of extract for each adsorption) and formalin-fixed Staph A. To bind the Con A–receptor complexes specifically, affinity-purified rabbit antibodies to Con A (10 μg in 20 μl) were added, and after incubating the mixture for 30 min on ice, 100 μl of a 20% suspension of Staph A in Con A extraction buffer was added. After 30 min at 4°C with continuous mixing, the bacterial pellets with adherent complexes were centrifuged ($8000 \times g$, 15 sec), washed 3 times in Con A extraction buffer, and the receptors were eluted by either (*i*) three successive extractions with α-methylmannoside (150 μl containing 200 mM α-methylmannoside in Con A extraction buffer), each for 15 min at 37°C, or (*ii*) one extraction with the sample buffer used for electrophoresis (3–5 min in a boiling water bath). Approximately 50–65% of the radiolabeled components adsorbed by the pellets were extracted with α-methylmannoside and 80–90% were extracted with $NaDodSO_4$/PAGE sample buffer (17).

$NaDodSO_4$/PAGE and Autoradiography. Material eluted from the Staph A pellets were analyzed by polyacrylamide slab gel electrophoresis in 0.1% $NaDodSO_4$, under reducing conditions, using a discontinuous pH gradient (18). Autoradiographs were prepared from dried gels with XAR-5 x-ray film (Kodak).

Characterization of Con A Receptors. Radiolabeled Con A receptors, eluted from Staph A-bound immune complexes with α-methylmannoside, were characterized by sequential immunoprecipitations with various mAb, using appropriate anti-Ig antibodies to facilitate binding to Staph A. The immunoprecipitated receptors were analyzed by $NaDodSO_4$/PAGE.

(γ-IFN) Production. CTL (clone 2C) 2×10^6 cells were incubated with 2.5 μg of Con A in 1.0 ml of RPMI 1640/0.1% albumin for 45 min at 37°C. After washing the cells 4 times the Con A–CTL were incubated for 20 hr in IFN-free medium. γ-IFN activity was determined in the harvested supernatant as described (19). To obtain T-cell growth factor free of γ-IFN, culture supernatants from Con A-stimulated AOFC cells were used (20).

Other Methods and Reagents. Previously described methods were used to generate conventional (polyclonal) secondary BALB.B anti-BALB/c CTL (13), to prepare ^{125}I-labeled Con A (8), and to remove bound Con A from Con A–CTL (8).

RESULTS

It was found previously that when Con A–CTL are incubated at 37°C they gradually lose their ability to recover cytotoxic activity after surface-bound Con A is eluted with α-methylmannoside (8). This change did not occur, or was very much slower, when the cells were incubated at 0°C. At the low temperature, full recovery of cytotoxic activity, after the removal of Con A with α-methylmannoside, persisted for at least 8 hr. The difference implies that there is a temperature-dependent Con A-induced loss of some of the surface receptors for Con A (modulation). Using FITC-labeled Con A we have observed that surface "patching" of Con A on the surface of cloned CTL does indeed occur at 37°C but not at 0°C.

Con A-Induced Modulation. To analyze these effects in greater detail, Con A–CTL that had been incubated for 4 hr at 37°C were compared with control Con A–CTL (incubated for the same time at 0°C) in regard to recovery of their cytotoxic activity after removing Con A from the cell surface. In parallel, the same cell populations were subjected to fluorescence-activated cell sorter analysis using either RITC-labeled Con A or a rat mAb to one of the structures identified below as a Con A receptor (T200) plus FITC-labeled goat antibodies to rat Ig.

A representative result (Fig. 1*A*) confirms the previous finding that after 4 hr at 0°C Con A–CTL recover virtually all of their initial cytolytic ability when treated with α-methylmannoside, whereas after the same time at 37°C removal of surface Con A results in the recovery of only about half the cytotoxic activity (measured in lytic units). In parallel with this loss of function at 37°C, there was a decrease of about 50% (compared to control Con A–CTL at 0°C) in the fluorescence intensity due to cell-associated RITC–Con A (Fig. 1*B*). There was also a similar loss at 37°C of a Con A receptor, revealed by decreased binding of the FITC-labeled anti-Ig that bound to anti-T200 antibody (Fig. 1*C*). Thus, Con A-induced loss (modulation) of a cell surface glycoprotein was associated with loss of cytotoxic activity. We define the involved glycoproteins as Con A receptors because they are evidently tightly bound by Con A on Con A–CTL.

Con A Receptors. To identify the Con A receptors, cloned CTL (G4, B10, 1D, 2C, cr 15) were radiolabeled, incubated with Con A, washed thoroughly, lysed with extraction buffer, and the labeled Con A receptors were isolated as complexes with Con A by immunoprecipitation with anti-Con A antibodies and Staph A. The receptors were then isolated from the sedimented complexes by elution with α-methylmannoside and visualized in autoradiographs of $NaDodSO_4$/polyacrylamide slab gels.

As shown in Fig. 2, only eight or nine labeled components were generally seen, whereas in controls prepared by adding Con A and anti-Con A antibodies to lysates of radiolabeled CTL, many more labeled components (total Con A receptors) were evident, in agreement with Henkart and Fisher (21) (compare lanes A and B). Usually, α-methylmannoside released 50–65% of the radiolabeled material from the Staph

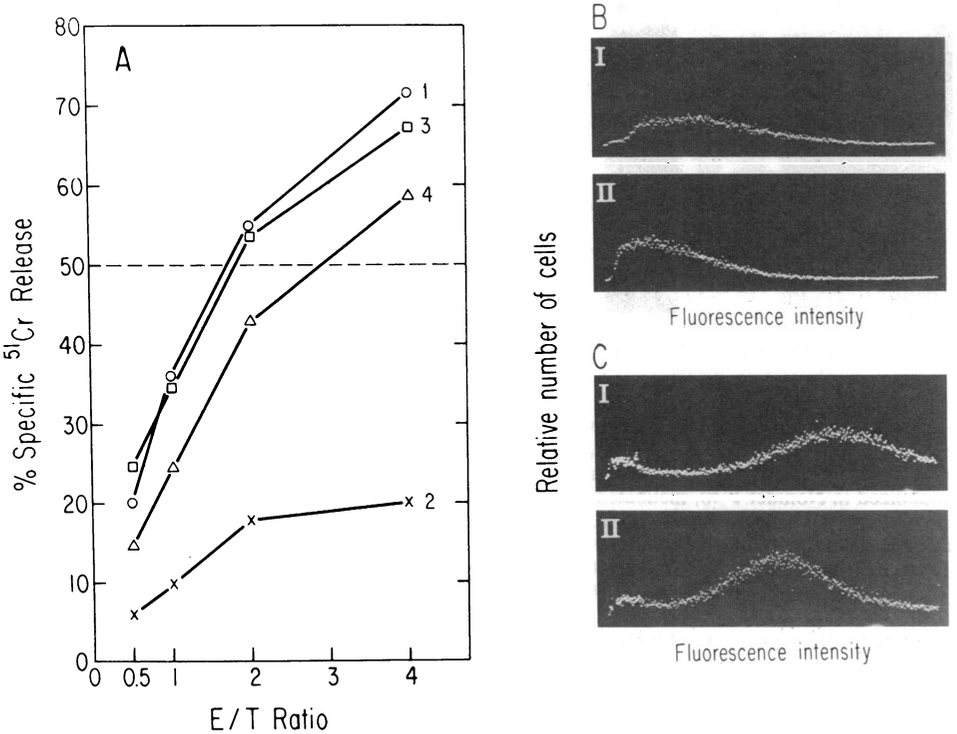

FIG. 1. Correlation between the loss of cytotoxic activity and the loss of Con A-binding receptors from the surface of CTL due to Con A-induced modulation. (*A*) Effect of temperature on recovery of CTL activity after treatment of Con A–CTL with α-methylmannoside. CTL, clone G4; target cells, P815. Curve 1, control (no pretreatment of CTL with Con A). Curve 2, CTL were pretreated with Con A (2.5 μg/ml, 1 hr at 37°C) and washed thoroughly to remove unbound Con A. The resulting cells (Con A–CTL) were then maintained for 4 hr at 37°C, washed with medium (no α-methylmannoside), and assayed. (The same results were obtained when the cells were kept 4 hr at 0°C.) Curve 3, Con A–CTL were kept at 0°C for 4 hr, then washed with α-methylmannoside and assayed. Curve 4, Con A–CTL were kept at 37°C for 4 hr, then washed with α-methylmannoside and assayed. (*B*) Loss of cell-associated RITC–Con A from Con A–CTL (clone G4) during 4 hr incubation at 37°C. (*I*) Fluorescence of cell-bound RITC–Con A after incubating Con A–CTL (clone G4) for 4 hr at 0°C. (*II*) Fluorescence of cell-bound RITC–Con A after incubating Con A–CTL for 4 hr at 37°C. (*C*) Loss of a Con A-binding receptor (T200) from RITC–Con A–CTL during 4 hr incubation at 37°C. After 4-hr incubation with RITC–Con A at 0°C (*I*) or 37°C (*II*), cells were treated with α-methylmannoside to remove cell-bound RITC–Con A and then incubated with monoclonal anti-T200 rat antibody and FITC-labeled goat antibodies to rat Ig. (*I*) Fluorescence of FITC-labeled reagent reveals T200 on the surface of RITC–Con A–CTL that were kept for 4 hr at 0°C. (*II*) Fluorescence of FITC-labeled reagent reveals T200 on the surface of RITC–Con A–CTL that were kept for 4 hr at 37°C. In control experiments, there were no significant losses of H-2^b after Con A-induced modulation on Con A–CTL.

A pellets; the residual material, extracted almost completely by heating the pelleted Staph A in NaDodSO$_4$/PAGE sample buffer in a boiling water bath, contained the same components that were seen in the α-methylmannoside extracts (compare lane B with lane C). The most prominent components (bands 1 and 2) were barely resolved in 7.5% gels and not at all on 10% gels (compare lanes B and L). Bands 3, 4, and 5 were of moderate intensity. The other bands were seen only after prolonged exposure of the gels (lanes J and K).

Some of the components released by α-methylmannoside from the Con A–anti-Con A antibody complexes were identified with monoclonal antibodies. As shown in Fig. 2 (lanes H and I), mAb to T200 precipitated band 1 (M_r, 190,000), and mAb to LFA-1 precipitated bands 2 and 5 (corresponding to the α- and β-chains of LFA-1 (6). On some of CTL clones (e.g., clone G4) mAb to Lyt-2 precipitated band 8. The band with M_r 25,000 (band C, lane K) represents radiolabeled Con A. Band 9 is an unidentified protein with M_r similar to that of Thy-1, but it is not precipitated by mAb to Thy-1.

To estimate the extent to which the various receptors are actually associated with Con A on the surface of intact Con A–CTL, the cell lysates were subjected to sequential precipitations, first with antibodies to Con A and then with mAb to the appropriate cell-surface antigens. The results indicated that virtually all T200, about one-half of LFA-1, and less than one-half of the Lyt-2 molecules on intact Con A–CTL form stable complexes with Con A (added to the cells at 2.5 μg of Con A per ml). Generally, the same pattern of Con A-binding receptors seen with ^{125}I-labeled CTL was observed with cells that were labeled metabolically—i.e., with [^{35}S]methionine, mixtures of ^{3}H-labeled amino acids and [^{3}H]mannose (data not shown).

Is it possible that some of the components that are immunoprecipitated from Con A–CTL by anti-Con A antibodies are not themselves Con A-binding glycoproteins but rather are associated tightly with such glycoproteins (i.e., with the "real" Con A receptors)? To answer this question the ^{125}I-labeled materials from bands 1–9 were eluted from gels, and their ability to bind to Con A-Sepharose 4B beads was tested. Over 80% of ^{125}I from bands 1, 2, 3, 4, 5, and 9 and 50% of labeled material from bands 6 and 7 were strongly bound to Con A-Sepharose.

DISCUSSION

In this study we have emphasized some properties of Con A–CTL, which are CTL that are prepared by incubating the cells with Con A at low concentrations (1–5 μg/ml) and then washing them extensively to leave on the cell surface only Con A that is tightly bound to what are tentatively designated as "high affinity Con A-receptors." In comparison with untreated CTL, these modified cells are greatly altered in their functions: their cytotoxic activity is dramatically decreased (Fig. 1*A*; see ref. 8), and they produce γ-IFN in the

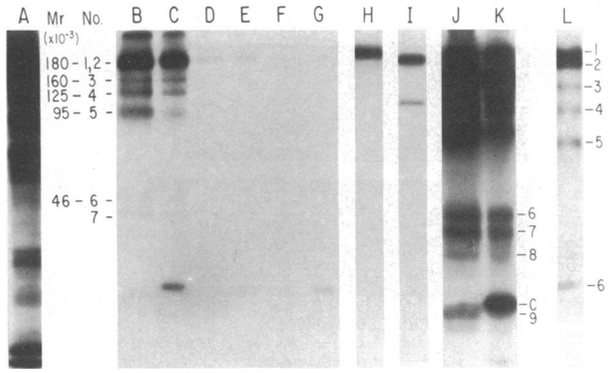

FIG. 2. Immunoprecipitation and NaDodSO$_4$/PAGE of Con A receptors from ^{125}I-labeled CTL (clone G4). All lanes were from 10% gels except lane L (7.5% gel). Lane A, total cell-surface Con A-binding glycoproteins revealed by the following: Ficoll-purified ^{125}I-labeled CTL were solubilized in Nonidet P-40, incubated with Con A at 2.5 μg/ml and immunoprecipitated with anti-Con A antibody and protein A-bearing Staph A. Lane B, high-affinity Con A-receptors revealed by the following: Ficoll-purified ^{125}I-labeled CTL were treated with Con A at 2.5 μg/ml and washed to removed unbound Con A; the resulting cells (Con A–CTL) were solubilized in Nonidet P-40 and immunoprecipitated with anti-Con A antibody and protein A-Staph A. Con A-binding glycoproteins were extracted from the immune complexes with α-methylmannoside and analyzed. Lane C, same as lane B but analysis of the immunoprecipitated material that remained on the Staph A pellets after α-methylmannoside extraction. Lane D, control in which ^{125}I-labeled Con A–CTL were incubated with 100 mM α-methylmannoside to remove Con A. Con A-receptors were then isolated as described in lane B. Lane E, same as lane D but analysis of the immunoprecipitated material that remained on the Staph A pellets after α-methylmannoside extraction. Lane F, control in which CTL were incubated with Con A (2.5 μg/ml) in the presence of α-methylmannoside (50 mM) and subsequently proceeded as in lane B. Lane G, same as lane F but analysis of the immunoprecipitated material that remained on the Staph A pellets after α-methylmannoside extraction. Lane H, Con A-receptors isolated as described in lane B were immunoprecipitated with anti-T200 mAb. Lane I, Con A-receptors isolated as described in lane B were immunoprecipitated with anti-LFA-1 mAb. Lane J, overexposed lane B. Note bands 6, 7, 8, and 9. Lane K, overexposed lane C. "C" points to the ^{125}I-labeled Con A that remained on the pellets. Lane L, Con A-receptors isolated as described in lane B were run on a 7.5% gel.

absence of antigen (Table 1). These pronounced changes are all evidently due to the tightly bound Con A, as they are reversed by eluting Con A from the CTL surface by α-methylmannoside. Because CTL normally appear to secrete γ-IFN only in response to specific interactions with their target cells (19), all of these observations suggest that the Con A receptors include surface molecules that are intimately involved in antigen recognition and effector functions of CTL.

Because of the relative stability of the complexes formed by Con A with the receptors we were able to develop a procedure, based on the use of anti-Con A antibodies, to isolate these receptors. The receptors were visualized as only 8 or 9 components on NaDodSO$_4$/PAGE and some of them could be accounted for by immunoprecipitation with mAb that are known to block CTL activity (5, 6). Thus mAb to LFA-1 precipitated bands 2 and 5, which correspond to LFA-1 α- (band 2) and β- (band 5) chains and anti-Lyt-2 antibody precipitated band 8 (on some clones).

A fourth component, the most intense band (band 1), was completely immunoprecipitated by mAb to T200. Although antibodies to T200 do not block CTL activity, studies reported elsewhere (11) suggest that T200 may play a role in the cytotoxic activity of CTL. In these studies tosyllysyl chloromethyl ketone, a well known serine protease inhibitor, has been shown to block CTL activity probably by virtue of its

Table 1. Binding of Con A by receptors on cloned CTL induces secretion of γ-IFN

Experimental conditions	γ-interferon in culture supernatant, units/ml*
CTL†	<6
CTL and Con A (2 μg/ml)	12,288
Con A–CTL	12,888
Con A–CTL and α-methylmannoside‡	<6

*One unit of γ-IFN is defined in ref. 19.
†Clone 2C cells were incubated in 80% RPMI 1640/20% conditioned medium (2 × 10^6 cells per ml). The conditioned medium was the culture supernatant of the AOFS (20) cells grown 48 hr in the presence of Con A (5 μg/ml). The supernatant contained T-cell growth factor (IL-2) but no γ-IFN (19). Residual Con A was removed from the conditioned medium by adsorption on Sephadex G-50. The CTL were incubated 20 hr and γ-IFN was assayed as described (19).
‡Con A–CTL were treated with α-methylmannoside as described (8).

affinity-labeling of T200. It is of interest in this connection that mAb to T200 do block the cytotoxic activity of natural killer cells (22) and that cloned CTL under some culture conditions can exhibit killing properties of natural killer cells (23).

The unidentified Con A receptors of M_r 160,000 (band 3), 125,000 (band 4), 46,000 (band 6), and 23,000 (band 9), have not previously been described. Whether they are involved in CTL function is not known. One or more of these unidentified components may be involved in the induction of γ-IFN production, because antibodies to T200, LFA-1, and Lyt-2 separately and in combination fail to induce γ-IFN production by CTL.

There are several possible artifacts that could lead to under- or overestimating the number of Con A receptors. The possibility that glycoproteins from fetal calf serum are adsorbed by CTL and misconstrued to be Con A receptors was ruled out because all of the Con A receptors identified with ^{125}I-labeled CTL could also be identified as a result of a metabolic labeling of the cells with [^{35}S]methionine, ^{3}H-labeled amino acids. The possibility that some of the occupied Con A receptors were lost by endocytosis or shed during preincubation with Con A seems unlikely for two reasons. First, the pretreatment period was too brief (30–45 min); second, pretreatment with Con A at 0°C gave the same pattern of Con A receptors as did the pretreatment at 37°C.

To deal with the possibility that some of the Con A receptors were dissociated from Con A during cell lysis with detergent, we have used a photosensitive crosslinking reagent coupled covalently to Con A. The results show that the same set of cell-surface proteins (bands 1–9 in Fig. 2) were crosslinked to Con A after light exposure was used to photoactivate the crosslinking reagent on Con A (unpublished observations).

It seems clear, therefore, that the labeled components shown in Fig. 1 represent the surface proteins on CTL that form extremely stable complexes with Con A and that some of them are involved in the effector function and activation of CTL.

Identification of LFA-1 as one of the Con A receptors on T-cell surface helps to explain some of the well known effects of Con A on lymphocytes. For example, Con A can both induce IL-2 release from alloreactive cloned T cells and inhibit proliferation of these cells (24). The paradoxical inhibition of proliferation is probably due to interaction of Con A with LFA-1, because antibodies to LFA-1 are known to inhibit T-cell proliferation (25).

The approach described here provides opportunities to isolate functionally interesting T-cell surface proteins with-

out impairing their functional properties, because elution with α-methylmannoside of immunoprecipitated material is much milder than other methods of dissociating antigen from antibodies in immunoprecipitates.

We thank Ms. Joan McDowell of the M.I.T. Cell Sorter Laboratory for her help in performing fluorescence-activated cell sorting. The unpublished observations on photoactivated cross-linking of Con A to Con A receptors were carried out with the expert help of Dr. Martin Schwartz. This work was supported by Research Grants CA-15472 and CA-28900, Training Grant 5-T32-CA-09255 and Center Grant CA-14051 to the Center for Cancer Research, Massachusetts Institute of Technology, from the National Cancer Institute.

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