ANTIGEN-SPECIFIC T LYMPHOCYTE CLONES

I. Characterization of a T Lymphocyte Clone Expressing Antigen-specific Suppressive Activity*

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Analyses of thymus-derived (T) lymphocytes that induce or suppress antibody responses have shown that these functions are mediated by sets of T lymphocytes that express different and characteristic patterns of cell surface glycoproteins (1–3). Examination of these specific T cell functions have also indicated that soluble mediators, or factors, can mimic the functions of intact T-suppressor or T-inducer cells (4–14). However, analysis of these materials has not yet permitted a clear insight into the structural basis of antigen-specific T cell function.

A direct approach to this question requires large numbers of homogenenous T cells for biochemical studies. So far, T cell lymphomas have not proved so useful as B cell myelomas (15, 16) because many do not grow well in culture and few express immunological function (17, 18). Hybrids resulting from fusion of T cells with tumor cells (19–24) usually express the phenotype of the tumor cell partner and tend to lose chromosomes in cell culture. Analysis of immunologically active T-cell hybrids has also suggested that with few exceptions (23), these cells synthesize extremely small amounts of antigen-specific material (19, 21).

Using a method that allows production of large numbers of antigen-specific inducer or suppressor T cells, we have found that all Ly-1^+2^- clones so far examined carry inducer but not suppressor activity and synthesize a characteristic pattern of polypeptides. In contrast, Ly-2^+ clones mediate suppressor but not inducer activity and synthesize a characteristic set of proteins that differs from T-inducer cells (25).

We describe here a clone of Ly-2^+ suppressor T cells that expresses surface receptors specific for glycophorin from sheep erythrocytes (SRBC).[1] Supernates of this clone contain biosynthetically labeled 70,000-mol wt proteins that (a) specifically bind to SRBC and (b) specifically and completely suppress primary anti-SRBC responses.

Materials and Methods

Animals. C57BL/6 (B6) and BALB/c mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

[1] *Abbreviations used in this paper:* B6, C57BL/6 mice; BRBC, burro erythrocytes, BSA, bovine serum albumin; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; HG, glycophorin peptides from human erythrocytes; HRBC, horse erythrocytes; mc, monoclonal; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; PFC, plaque-forming cells; SDS, sodium dodecyl sulfate; SG, glycophorin peptides from sheep erythrocytes; SRBC, sheep erythrocytes; TNP, 2,4,6-trinitrophenol.

Antisera.   Lyt-1.2, Lyt-2.2, and Ly-5.1 antisera, prepared as described elsewhere (26, 27), were kindly donated by Dr. F.-W. Shen Memorial-Sloan Kettering Cancer Center, New York; monoclonal (mc) anti-Thy-1-2 by Dr. Ed Clark, University of Washington, Seattle, Wash., and mc-anti Ly-1 and mc-anti Lyt-2 by Dr. J. Ledbetter and Dr. L. Herzenberg, Stanford University School of Medicine, Stanford, Calif. Expression of surface glycoproteins by each clone was determined by immunofluorescence using both mc antibodies and antisera as described previously (25, 28).

Antigens and Immunization.   SRBC, horse erythrocytes (HRBC), and burro erythrocytes (BRBC) were purchased from Colorado Serum Co., Denver, Colo. Mice were immunized i.v. with 2×10^8 erythrocytes in 0.25 ml balanced salt solution 1–3 wk before use. Glycophorin from SRBC and HRBC (>60% homogeneous) were obtained by eluting the corresponding bands from sodium dodecyl sulfate (SDS) polyarcylamide gels of ethanol-extracted erythrocyte membranes. The asialo form of sheep glycophorin was obtained by neuraminidase treatment of the eluted glycophorin. CH-2 peptide, (>80% homogeneous) corresponding to the N-terminal region of sheep glycophorin, was obtained after chymotryptic digestion of SRBC and purified as described elsewhere (29). Human erythrocyte glycophorin as well as its N-terminal tryptic peptide, T-2, (amino acids 1–33) were purified to virtual homogeneity as described previously (29, 30).

Preparation of Lymphoid Cell Populations

$Ly-1^+2^-$ CELLS.   Spleen cells from erythrocyte-immune donors were passed through nylon-wool columns according to the method of Julius et al. (31). Ig^-, $Ly-1^+2^-$ cells were positively selected from this cell population as follows (32, 33): Fisher petri dishes (Fisher Scientific Co., Pittsburgh, Pa.) were incubated with mc-anti-Ly-1 (12 µg/dish) and goat Ig (640 µg/dish) in 10 ml for 12 h before extensive washing with phosphate-buffered saline (PBS)-2% fetal calf serum (FCS); 5 ml of column-passed cells (10^7/ml) were incubated in these dishes for 60 min at 4°C with occasional shaking. After removal of nonadherent cells by swirling the plate gently, 10 ml RPMI-1640 supplemented with 10% FCS was added and the plates were slowly rocked for an additional 30 min at 37°C. After gentle pipetting, eluted cells were washed twice and incubated with anti-Lyt-2.2 and Lyt-3.2 (3×10^7 cells/ml; final concentration of anti-Lyt sera: 1:30) followed by incubation for 40 min at 37°C with selected rabbit serum as a source of complement. This procedure resulted in a cell population ≥98% $Lyt-1^+2^-$, Ig^- according to immunofluorescence using either mc-anti-Ly antibodies or conventional anti-Ly sera.

B CELLS.   After positive selection of cells on anti-Fab-coated plates (33), eluted cells were incubated with mc-anti-Thy-1.2 (final dilution: 1:1,000), anti-Lyt-1.2, and anti-Lyt-2.2 (final dilution of each: 1:30) for 35 min on ice, washed, and incubated an additional 30 min with rabbit complement. Lack of residual T cells was verified by immunofluorescence using mc-anti-Thy-1 antibody, as well as by the absence of anti-SRBC plaque-forming cell (PFC) responses after in vitro stimulation by SRBC.

Biological Assays for Suppressive Activity.   The ability of supernates of cloned T cell populations to inhibit in vitro generation of anti-SRBC PFC was determined by adding supernate material to mixtures of 10^5 Ly-1 cells from SRBC-immune donors and 10^6 B cells from nonimmune donors in a final vol of 0.2 ml. These cell cultures were stimulated with 10^6 SRBC for 5 d before anti-SRBC PFC were enumerated (34). Inhibition of anti-SRBC helper function was tested by preincubating 10^5 Ly-1 cells from SRBC-immune donors (obtained as described above) with different supernate preparations for 24–36 h. Cells were then washed three times with PBS-2% FCS, counted and tested for SRBC-specific helper function after addition to cultures containing 10^6 B cells and SRBC. In all cases, anti-erythrocyte PFC and total PFC were determined from triplicate cultures (35–37).

Generation of Cl.Ly23/4 Cells.   T cells from the B6 mice immunized with SRBC were treated with anti-Lyt-1.2 and complement twice before incubation in plastic dishes coated with sheep glycophorin (10 µg/dish). Adherent cells were released by vigorous pipetting, distributed into microwells (Falcon 3040 microtiter plates; Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.), and expanded using culture conditions that have been described elsewhere (25). The Cl.Ly23/4 line was derived from a colony that arose in wells seeded with 1–10 cells; after 2 mo of growth, the clone was derived by micromanipulation of a single cell (25).

Binding of Soluble Antigen to Cloned Cell Populations.   Cloned cell populations were washed three

times in PBS-2% FCS and resuspended at 5×10^5 cells/ml in the same medium. After incubation with various radiolabeled antigens at 4°C for 1 h, the cells were washed five times in PBS-2% FCS and radioactivity in the cell pellet was determined.

Internal Labeling of Cloned Cells. Production of labeled supernatant material from cloned cells was carried out in serum-free conditions as follows: cells from continuously growing T cell clones were washed three times and incubated at 10^6 cell/ml in methionine- and glutamine-free RPMI-1640 medium that was supplemented with 2 mM glutamine, 5 µg/ml human transferrin, 5 µg/ml bovine insulin, 20 U/ml aprotinin and 100–200 µCi [^{35}S]methionine (1,000 Ci/mmol). After 4 h at 37°C, unlabeled methionine was added to bring the final concentration of methionine to 1 mM. Cells were pelleted by centrifugation, washed repeatedly with PBS, and stored at −70°C for further use. 0.1 mg/ml ovalbumin was added to the supernate as carrier protein before centrifugation at high speed (100,000 g for 1 h) before use.

Estimation of the Amount of Protein in Cl.Ly23/4 Supernatant Fractions. The approximate amount of protein in different supernatant fractions was estimated from the radioactivity of the samples according to the following assumptions: (a) methionine = 1% protein content; (b) 10^6 cpm methionine = 1 µg protein. Because intracellular methionine levels can affect the above estimates, cells were routinely pulsed with [^{35}S]methionine of different specific activities to determine the degree of dilution of radiolabeled methionine. Because the results of these experiments varied up to one order of magnitude the figures of protein concentration given in this report are accurate within a single order of magnitude, and are most useful for comparison purposes within a single experiment.

Binding of ^{35}S-labeled Supernatant Material to Erythrocytes. SRBC, HRBC, or BRBC (10^7/ml) were incubated with supernatant material from different T cell clones for 2 h at 4°C. The cells were pelleted by centrifugation (1,000 g), washed twice with PBS, and when relevant, dissolved in Laemmli sample buffer (38) for subsequent analysis on polyacrylamide gels or scintillation counting.

Affinity Chromatography of Clone Cl.Ly23/4 Supernate. Different glycophorins were conjugated to Sepharose 4B with cyanogen bromide (2 mg protein/ml beads) (39). Cell supernate material or serum-free media (see above) was mixed with the immunoadsorbent beads (0.1 ml beads/ml supernate) in 5-ml syringe columns before incubation for 2 h at 4°C with occasional shaking. The columns were then extensively washed with PBS, followed by elution with Sorensen's glycine-HCl (pH 2.4) buffer and immediate neutralization with excess Tris-HCl (2 M, pH 7.2). The suppressive activity of eluted cell supernatant material was always compared with the effects of serum-free media that had been eluted under identical conditions (buffer control).

Polyacrylamide Gel Electrophoresis. Unless otherwise indicated, electrophoresis was carried out in 0.7-mM thick slab gels using a modification of the method of Laemmli (38). Briefly, 25-µl aliquots of the samples for analysis were mixed with an equal volume of a solution containing 160 mM Tris-HCl, pH 6.8; 20% glycerol; 4% SDS; 0.4% bromophenol blue; and the material was boiled for 5 min. When reducing conditions were desired, 200 mM dithiothreitol was added. Gels containing 12.5% acrylamide and 0.1% SDS were subjected to electrophoresis at 120 V constant voltage until the tracking dye reached the bottom of the gel. The gels were stained with Coomasie Blue and destained with 25% isopropanol-10% acetic acid solution. The method of fluorographic treatment of Laskey and Mills (40) was routinely used to enhance gel sensitivity and to reduce exposure time before drying. Radioautographs obtained using Kodak XP-Omat XR-5 films (Eastman Kodak Co., Rochester, N. Y.) were scanned in a Joyce-Loebl densitometer (Joyce, Loebl and Co., Gateshead-on-Tyne, England).

Results

Generation of Clone Cl.Ly23/4. T cells from SRBC-immune B6 mice were treated with anti-Lyt-1.2 plus complement and eluted from dishes coated with SRBC glycophorin before initiation of clonal growth. Clones were screened by testing metabolically labeled supernates for (a) binding to SRBC, HRBC, or human erythrocytes and (b) inhibition of in vitro production of anti-SRBC PFC by mixtures of Ly-1 cells and B cells. A clone was considered positive if [^{35}S]methionine-labeled supernate polypeptides bound to SRBC but not HRBC or human erythrocytes and

inhibited the PFC response to SRBC but not HRBC or BRBC. One of eight clones tested, termed Cl.Ly23/4, was positive by both criteria.

The cell surface phenotype of Cl.Ly23/4 cells (determined by immunofluorescence) is Thy-1$^+$, Lyt-2$^+$, Ly-5$^+$, Ig$^-$, NK-1$^-$ and this phenotype has been stable for >12 mo of in vitro growth. Samples of these cloned cells are routinely frozen for storage; cells are grown from thawed samples to >10^8 without detectable change in surface phenotype, function, or pattern of polypeptide synthesis (25). Expression of Ly-1 is ambiguous: indirect immunofluorescence generally indicated that a portion of cells (10–30%) emitted fluorescence slightly above background levels on the fluorescence-activated cell sorter (FACS).

Clone Cl.Ly23/4 Expresses Receptors for SRBC Glycophorin (Fig. 1).   Cells from the Cl.Ly23/4 clone were tested for binding to ^{125}I-labeled chymotryptic peptide (CH2) that corresponds to the N-terminal or external portion of SRBC glycophorin (30). Cl.Ly23/4 cells bound the CH$_2$ peptide from SRBC glycophorin but did not bind the N-terminal portion of glycophorin from human erythrocytes (T-2) (Fig. 1 A). Other cloned T-cell populations did not bind either material (data not shown). Specificity of binding was verified by competition with different unlabeled glycophorins; excess intact cold glycophorin from sheep erythrocytes but not from erythrocytes of other species inhibited binding (Fig. 1 B).

Demonstration of Antigen-binding Peptides in Supernates of Cl.Ly23/4 Cultures (Tables I and II and Fig. 2).   Cloned cells were pulsed with [^{35}S]methionine for 4 h in serum-free media (Materials and Methods). After centrifugation (100,000 g), the supernate was incubated with different erythrocytes, (10^7/1 ml for 2 h at 4°C). 4–8% of total cpm present in the supernate of Cl.Ly23/4 cells bound to SRBC; <2% bound to HRBC or BRBC. Other cloned cell populations from SRBC-immune donors that expressed different cell surface phenotypes (Thy-1$^+$, Ly-1$^+$2$^-$; Thy-1$^+$, Ly-1$^-$2$^-$, NK-1$^+$) did not release antigen-specific binding proteins into the medium under the same conditions. Moreover, supernates from a cloned Thy-1$^+$, Ly-1$^-$2$^+$ cell population obtained from
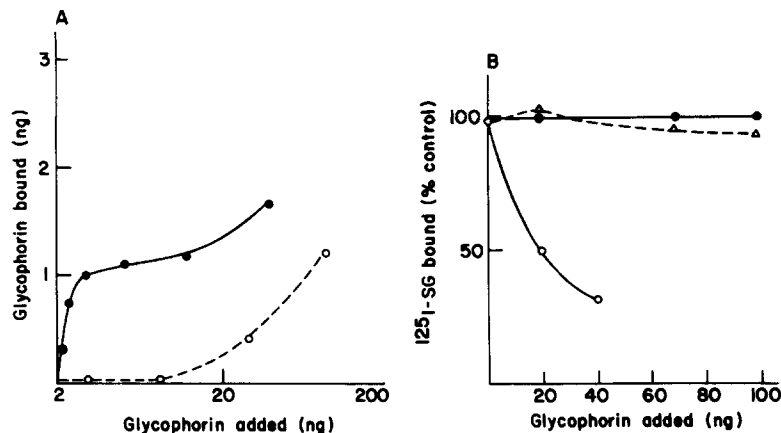

FIG. 1.   Binding of erythrocyte glycophorins to Cl.Ly23/4 cells (A) Saturation binding. 3 × 10^5 cells in 0.6 ml PBS-2% FCS were incubated with different amounts of ^{125}I-labeled chymomtryptic peptide (CH-2) from SG (●) or its HG equivalent, the T-3 tryptic peptide (○), for 1 h at 4°C. (B) Competition binding. 3 × 10^5 cells in 0.6 ml PBS-2% FCS were incubated with 20 ng ^{125}I-SG CH-2 for 10 min at 4°C before addition of the indicated amounts of unlabeled glycophorins from either SRBC (○) human erythrocytes (●) or HRBC (△) for 60 min.

TABLE I

Binding of [^{35}S]Methionine-labeled Supernates from Cloned Cells to Erythrocytes

Clone super-nate	Counts per minute bound				
	SRBC	HRBC	BRBC	TNP-BSA	BSA
Cl.Ly23/4	3,450	1,250	1,140	ND	ND
Cl.Ly23/11	6,220	1,700	1,340	ND	ND
Cl.NK11	310	770	ND	ND	ND
Cl.Ly1/4	1,760	1,940	ND	ND	ND
Cl.Ly23/18*	1,350	1,450	1,380	4,350	1,285

[^{35}S]Methionine internally labeled supernatant peptides (5 × 10^4 cpm) from different cloned populations was incubated with 10^7 erythrocytes (Materials and Methods) or added to a polyvinyl plate coated with bovine serum albumin (BSA) or TNP-BSA. Although 2% of the counts per minute in supernates of Cl.Ly23/4 bound to BRBC or HRBC, no discrete internally labeled peptide was detected after electrophoresis of ~5,000 cpm of BRBC or HRBC-bound material on polyacrylamide gels, in contrast to polyacrylamide gel electrophoresis of 5,000 cpm of SRBC-bound material (Fig. 2).
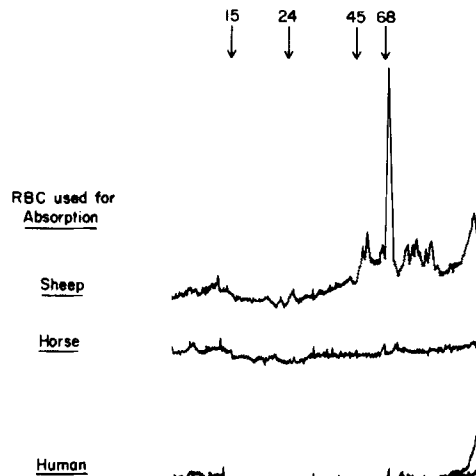* TNP-specific clone.
‡ Not done.

FIG. 2. SDS-polyacrylamide gel electrophoresis of Cl.Ly23/4 supernatant material bound to erythrocytes. [^{35}S]Methionine-labeled supernate (10^5 cpm) from Cl.Ly23/4 cells was incubated with 10^7 of the indicated erythrocytes (RBC). After washing twice with PBS, erythrocyte-bound material was dissolved in Laemmli sample buffer and 5,000 cpm of each subjected to electrophoresis in 12.5% acrylamide gels. Densitometric scans of the radioautographs of these gels after fluorography treatment are shown. 15, 24, 45, 68 = 15,000, 24,000, 45,000, 68,000 mol wt, respectively.

donors immune to a different antigen 2,4,6-trinitrophenyl (TNP) bound TNP but not SRBC. (Table I).

Internally labeled supernates from Cl.Ly23/4 bound to SRBC, HRBC, or human erythrocytes (5 × 10^3 cpm each) were analyzed by electrophoresis in SDS-polyacrylamide gels under reducing conditions. Densitometric scanning of the radioautographs of the bound material demonstrated that a major polypeptide of ~70,000 mol wt bound SRBC (Fig. 2). No discrete polypeptide was detected in material that adhered

TABLE II

Effect of Different Glycophorins on Binding of Internally Labeled Cl.Ly23/4 Supernate to SRBC

Glycophorin	Amount added	[35S]Methionine super-nate bound		Percent inhibi-tion specific cpm bound to SRBC
		SRBC	BRBC	
	μg/ml			
None	—	1,600	540	Standard
SG	10	498	583	100
SG	1	1,410	ND	18
Horse glycophorin	10	1,471	637	12
SG (asialo)	10	1,206	ND	36
SG (CH-2 peptide)	4	830	ND	70
HG (T-3 peptide)	4	1,567	ND	3

[35S]Methionine-labeled Cl.Ly23/4 supernatant proteins (2 × 10^4 cpm in 0.5 ml) were incubated with 5 × 10^6 SRBC or BRBC in the presence of the indicated amount of glycophorins. The specific counts per minute bound to SRBC were obtained by subtracting the counts per minute bound to BRBC (540) from the counts per minute bound to SRBC.

TABLE III

Cl.Ly23/4 Clone Supernate Suppresses Anti-SRBC PFC Responses

Dilution of Cl.Ly23/4 supernate	Anti-SRBC PFC/10^6 cells	Percent-age of control	Total PFC/10^6 cells	Percent-age of control
No supernate	2,011 ± 140		1,032 ± 48	
10^{-2}	0 ± 0	0	451 ± 24	45
10^{-3}	315 ± 28	16	892 ± 35	88
10^{-4}	1,307 ± 85	52	1,038 ± 59	100

Supernate from Cl.Ly23/4 cells was added to a mixture of 10^6 SRBC-immune Ly-1^+2^- helper T cells, 10^6 nonimmune B cells, and 10^6 SRBC. Anti-SRBC PFC and total PFC were enumerated 5 d later. Results are shown as the mean PFC ± SE of triplicate cultures (Materials and Methods).

to HRBC or human erythrocytes despite 2 wk of exposure of gels containing the same total counts per minute as SRBC-bound material.

Binding of ^{35}S-labeled supernatant material to SRBC was a result of a glycophorin recognition, because binding to 5 × 10^6 SRBC was inhibited by 10 μg of intact sheep glycophorin or 4 μg of the CH-2 peptide, but was not inhibited by human glycophorin (Table II). This inhibition cannot be attributed to nonspecific properties of glyco-phorin molecules from sheep erythrocytes (e.g., increased hydrophobicity) because background levels of cpm bound to another erythrocyte, BRBC, were not affected by addition of SRBC glycophorin. Sugar residues present on the glycophorin molecule apparently play an important role in its antigenicity because neuraminidase treatment of sheep erythrocyte glycophorin greatly reduced its ability to inhibit binding (Table II).

SRBC Glycophorin-specific Supernatant Molecules from Cl.Ly23/4 Suppress the Anti-SRBC Response (Table III, Figs. 3–5). Highly diluted supernates obtained from 10^6 Cl.L623/4 cells inhibited the production of anti-SRBC Ig but not total Ig secretion by mixtures of Ly-1 cells and B cells. However, higher concentrations of supernate also inhibited
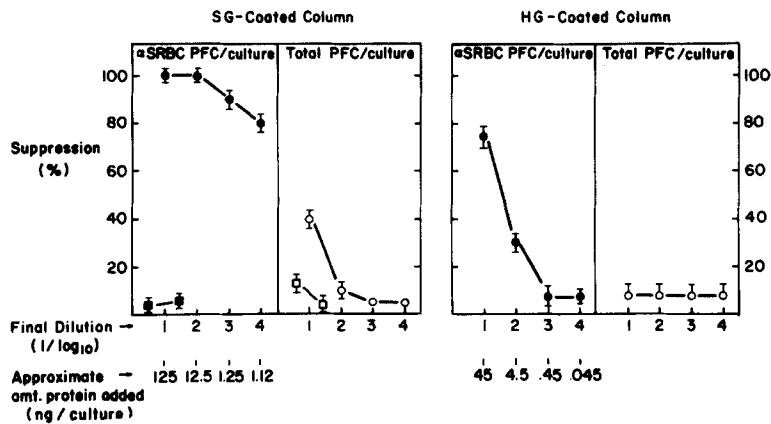
FIG. 3. Suppressive activity of Cl.Ly23/4 supernate after elution from SG or HG columns. Cl.Ly23/4 supernate (10 ml) was applied to 1-ml glycophorin columns. After intensive washing with PBS, passed (□, ■) and acid-eluted material (●, ○) were tested for suppression of in vitro anti-SRBC (αSRBC) PFC and total PFC responses. Results are shown as the mean suppression ± SE. PFC responses incubated with buffer controls were 850 ± 60 anti-SRBC PFC and 10,530 ± 795 total PFC. amt, amount.

total Ig secretion as measured by a reversed PFC assay (Table III). To determine whether antigen-binding molecules were responsible for this suppressive activity, supernates of Cl.Ly23/4 cultures were applied to Sepharose 4B columns coupled with glycophorin from SRBC (SG) or human erythrocytes (HG).

Suppressive activity of material retained on SG- or HG-coated columns was quantitated by adding graded amounts of the eluted protein (estimated from [^{35}S]-methionine content; Materials and Methods) to SRBC-stimulated cultures. As little as 1 ng of material eluted from SG-coated columns inhibited 75% of the anti-SRBC PFC response (Fig. 3); suppressive activity was not detectable in supernate that did not adhere to SG-coated columns. Material eluted from HG-coated columns contained suppressive activity at high concentrations (50 ng/ml) (Fig. 3). This latter activity might be accounted for by cross-reactive binding to HG, or by nonspecific interactions with HG. We favor the latter explanation because suppressive activity retained on HG-columns but not on SG-columns could be eluted by Nonidet P-40 (NP-40) nonionic detergent (0.3%) (Fig. 4). To determine whether suppressive activity in supernatant material that passed through HG-coated columns after washing with PBS could bind to SG, this material was reapplied to SG-coated columns (Fig. 5). These data show that passage of Cl.Ly23/4 supernate through HG-coated columns before elution from SG-coated columns resulted in approximately a 50-fold increase in specific suppressive activity/ng of protein: as little as 0.02 ng of this material inhibited 80% of the anti-SRBC PFC response.

Cellular Target of Clone Cl.Ly23/4 Suppressive Activity (Fig. 6). The above information indicates that material synthesized by Cl.Ly23/4 (*a*) binds to sheep erythrocyte glycophorin and (*b*) inhibits in vitro generation of anti-SRBC PFC by mixtures of Ly-1 cells and B cells. To determine which of these cell populations is the target of suppression, Ly-1 cells or B cells were preincubated with the SG-eluted fraction of Cl.Ly23/4 supernate for 36 h. After washing four times, Ly-1 cells were tested for helper activity and B cells were tested for antibody-forming activity. Preincubation of
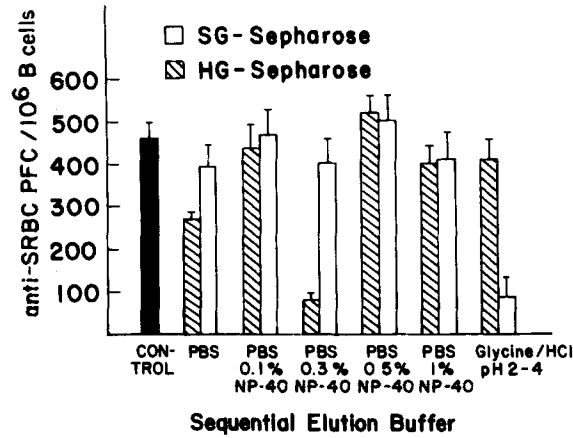
FIG. 4.   Effect of low concentrations of NP-40 on the retention of suppressive activity on glycophorin columns. CL.Ly23/4 supernates were applied to glycophorin columns as described in Fig. 3. The columns were then washed sequentially with 10-ml aliquots of PBS containing increasing amounts of NP-40 (0–1%) followed by final elution with 10 ml glycine-HCl, pH 2.4, buffer. The different fractions (after dialysis against PBS) were tested for suppression of anti-SRBC PFC at a final dilution of (1:20).
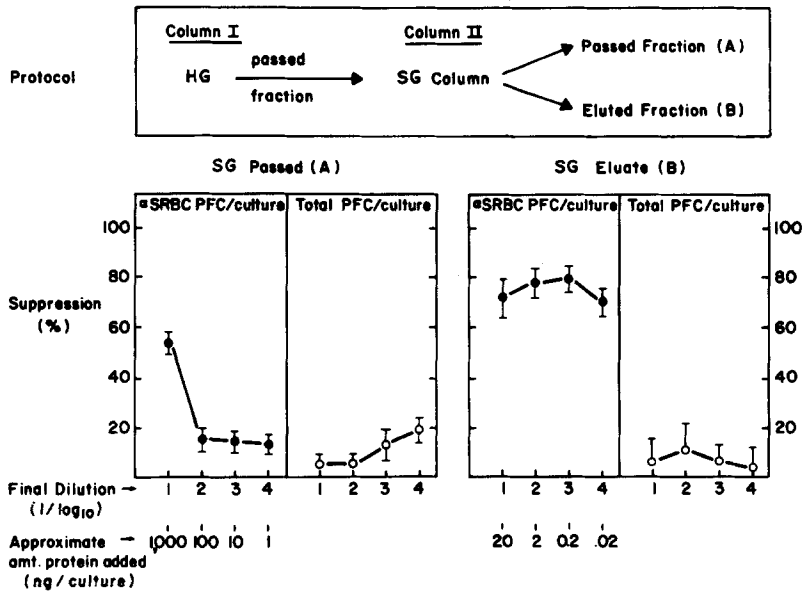

FIG. 5.   Suppressive activity of Cl.Ly23/4 supernate passed through columns with HG binds to columns coated with SG. CL.Ly23/4 supernate was passed through HG-coated columns before application to SG-coated columns. SG-coated columns were washed with PBS (passed fraction) before elution with low pH buffer (eluted fraction). The passed and eluted fractions were tested for suppression of anti-SRBC (αSRBC) PFC or total PFC. PFC responses of cell cultures incubated with control buffer were 950 ± 35 anti-SRBC PFC and 7,850 ± 430 total PFC.

Ly-1 cells with Cl.Ly23/4 supernate material eliminated subsequent helper activity; preincubation of B-cells did not affect subsequent antibody-forming activity. In addition, this analysis demonstrates that suppression of Ly-1 cell helper activity by Cl.Ly23/4 supernate requires the presence of antigen (SRBC).
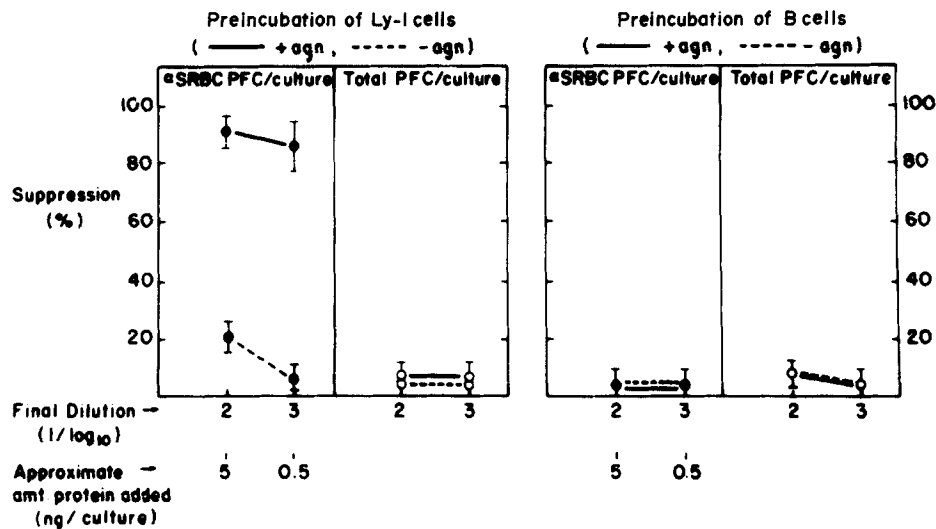
Fig. 6. Cellular target of Cl.Ly23/4 suppression and requirement for antigen (agn). Ly-1 cells from SRBC immune donors (10⁵) or B cells (10⁶) from unimmunized donors were preincubated with 5 or 0.5 ng of material eluted from SG-coated columns with or without 10⁶ SRBC for 36 h at 37°C. After washing the cells twice with PBS-2% FCS, 10⁵ Ly-1 cells were tested for anti- (α) SRBC helper T cell activity after addition to 10⁶ fresh B cells and 10⁶ SRBC. Preincubated B cells were tested for antibody-forming activity after mixing with 10⁵ Ly-1 cells from SRBC-immune donors and 10⁶ SRBC. αSRBC PFC and total PFC were enumerated 4 d later. Cultures containing Ly-1⁺2⁻ cells preincubated with buffer control produced 720 ± 80 anti-SRBC PFC and 8,640 ± 870 total PFC. Cultures containing B cells preincubated with buffer control produced 1,400 ± 85 αSRBC PFC and 8,930 ± 1,250 total PFC.
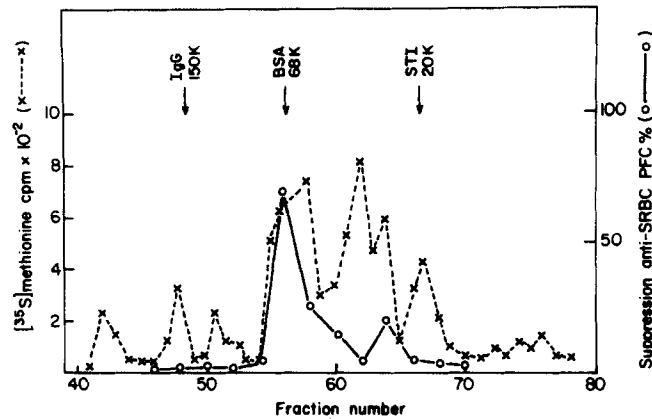

Fig. 7. Sephacryl S-200 column chromatography of SG-binding proteins from Cl.Ly23/4 supernates. Internally labeled Cl.Ly23/4 supernatant material was eluted from SG-coated columns by low pH buffer and fractionated by Sephacryl S-200 chromatography on 100- X 2-cm columns equilibrated with PBS containing 2% glycerol, 1 mM, phenylmethylsulfonylfluoride, and 100 mM KCl. Each 2-ml fraction was tested for radioactivity and for suppression (final dilution: 1:100) of the anti-SRBC PFC resource in cultures containing SRBC and mixtures of Ly-1⁺2⁻ cells and B cells. K, 1,000 mol wt; STI, soybean trypsin inhibitor.

TABLE IV

Specific Inhibition of Inducer T Cell Function by 70,000-mol wt Polypeptides That Bind to SG

Source of Ly-1 cells	70,000-mol wt fraction added	Anti-erythrocyte PFC/10^6 B cells	
		Anti-SRBC	Anti-HRBC
	ng		
SRBC-immune donors	0	2,758 ± 160	—
SRBC-immune donors	0.5	168 ± 30	—
HRBC-immune donors	0	—	2,800 ± 240
HRBC-immune donors	0.5	—	2,780 ± 330

Fractions 56–58 from Sephacryl S-200 column chromatography (Fig. 7) were pooled and concentrated. Ly-1^+2^- helper T cells (10^6) from either SRBC- or HRBC-immune donors were preincubated for 24 h with 0.5 ng of this material and either 10^6 SRBC or HRBC before testing for helper T cell activity as indicated Fig. 6.

A 70,000-mol wt Fraction of Cl.Ly23/4 Supernate Specifically Suppresses T Helper Activity for SRBC but No Other Erythrocytes (Fig. 7 and Table IV).   Because suppressive activity in Cl.Ly23/4 supernate was lost after passage through SG- but not HG-coated columns, we asked whether the molecular weight of suppressive material corresponded to the molecular weight of material that bound to SRBC. After Sephacryl S-200 chromatography of material eluted from SG-coated columns, suppressive activity was concentrated in a few fractions having the same molecular weight as antigen-binding material (Fig. 7, 70,000 mol wt).

To determine whether suppression of Ly-1 cells by the 70,000-mol wt supernate material was SRBC specific, Ly-1 cells from donors immune to SRBC or HRBC were preincubated with the homologous erythrocyte and the 70,000-mol wt fraction obtained after elution from Sephacryl columns; this incubation diminished subsequent anti-SRBC but not anti-HRBC helper activity (Table IV). Similarly, preincubation of Ly-1 cells from donors immune to BRBC did not inhibit subsequent anti-BRBC helper activity (data not shown).

Discussion

We have generated cloned cells bearing the Thy-1^+, Ly-$1^±2^+3^+$ surface glycoprotein pattern (Cl.Ly23/4). This cell line was derived by single cell micromanipulation and has been propagated in continuous culture for >12 mo. Cells of this cloned line have maintained stable expression of cell surface gene products (Ig$^-$Ly/b2$^-$; Thy-1^+; Ly-$1^±2^+$, Ly-3^+, Ly-5^+; NK-1^-, Qa-5^-), and stable biologic function. Cells of this clone, in contrast to T suppressor clones that had been derived after elution from dishes coated with other antigens, bound specifically to a chymotryptic peptide (CH-2) (Fig. 1), which represents the external portion of the SG molecule (29, 30). Because the CH-2 peptide does not contain amino acids that span the erythrocyte membrane, it is considerably less hydrophobic than the entire molecule. Because Cl.Ly23/4 cells bound the CH2 peptide at least as efficiently as the whole molecule, it is likely that binding reflects immunologic recognition of the external portion of the molecule rather than hydrophobic interactions. These data (Fig. 1 A and B) demonstrate that cloned T cells express specific and saturable receptor activity for free antigen; we do not know whether major histocompatibility complex products also contribute to the

affinity of this antigen-specific binding. Although we have not performed extensive binding studies, data obtained so far indicate that each T cell binds ~5 × 10^4 SG molecules. Assuming one glycophorin molecule bound per receptor, this figure is ~10-fold lower than the numbers of cell-bound receptors thought to be expressed on B cells (41).

This analysis also demonstrates that, in addition to carrying receptors for antigen at its membrane surface, Cl.Ly23/4 cells produce antigen-binding polypeptides (Table I). Although we have not formally demonstrated that these internally labeled polypeptides are secreted, the findings that (a) virtually all antigen-binding activity is present in the supernatant fraction after centrifugation at 100,000 g (Fig. 2) and (b) very little is detectable in membrane or cytoplasmic fractions (M. Fresno, L. McVay-Boudreau, and H. Cantor, manuscript in preparation) speaks against the possibility of binding by supernatant material randomly shed from the cell. Analysis of metabolically labeled material in supernates demonstrated that a molecular species having an apparent 70,000 mol wt (Fig. 2) accounted for almost all specific binding and that binding of this 70,000-mol wt material to sheep erythrocytes was completely inhibited by glycophorin-enriched peptides from SRBC but not human erythrocytes or HRBC (Table II).

Approximately 2–3% of internally labeled proteins synthesized by Cl.Ly23/4 cells is found in the supernatant fraction and ~10% of this represents antigen-binding material. By contrast, extensive analysis of clones of prethymic cells, thymocytes, inducer cells and NK cells has shown that these cell types do not synthesize 70,000-mol wt antigen-binding polypeptides (25). These findings suggest that synthesis and secretion of these antigen-binding 70,000-mol wt peptides is a specialized function of Cl.Ly23/4 cells.

The combined use of the sheep erythrocyte and different glycophorin molecules has allowed analysis of the fine specificity of binding by the 70,000-mol wt polypeptides. Binding to SRBC is likely to reflect recognition of glycophorin determinants because it is inhibited by low concentrations of intact molecules as well as the N-terminal portion of this molecule. The latter peptides inhibit less efficiently than the whole molecule. This may indicate the contribution of an additional sequence of the glycophorin to binding or may simply reflect differences in the conformation between the intact glycophorin and the chymocryptic peptide. Interestingly, sialic acid residues on glycophorin molecules play a major role in its recognition by both 70,000-mol wt peptides (Table II) and the intact cells (data not shown).

It is generally accepted that suppressor effector cells express the Ly-1$^-$23$^+$ surface phenotype (1–3, 42). After incubation with mc-Lyt-1 or conventional Ly-1 antisera, a portion of Cl.Ly23/4 cells (~20%) are read as slightly above background in the FACS. This does not reflect the surface phenotypes of two distinct clones because Cl.Ly23/4 was derived after single cell micromanipulation and regrowth (25). Ly-1$^+$2$^+$ cells in uncloned T cell populations may produce antigen-specific material (43) and have been implicated as precursors of suppressor cells (42). Because only about one-fifth of Cl.Ly23/4 cells express low amounts of Ly-1, an intriguing possibility is that cells within this clone may continuously differentiate in cell culture as follows: Ly-1$^+$2$^+$ (20%) → Ly-1$^-$2$^+$ (80%).

The results reported here demonstrate that internally labeled peptides from Cl.Ly23/4 specifically suppress in vitro antibody responses. These suppressive peptides

bind specifically to columns coated with SG (Figs. 3 and 5) and have an ~70,000 mol wt (Figs. 2 and 7). Although small amounts of suppressive activity are detectable in material bound to columns coated with HG, this binding is sensitive to NP-40 detergent, unlike binding to columns coated with SG.

The 70,000-mol wt polypeptides described here can be classified as final mediators of T suppression because they directly inhibit helper activity of Ly-1 cells. By contrast, most products from T suppressor cells that have been studied stimulate other T cells to augment T-suppression primarily by activating Ly-1$^+$2$^+$ cells to amplify the generation of suppression from Ly-1$^-$2$^+$ cells (4, 5, 7–12).

Perhaps the most striking finding reported here is that polypeptides from a single T cell clone can completely preempt the antibody response to a foreign cell. These studies do not rule out the possibility that suppression is carried out by a family of polypeptides, and that only a small fraction of these polypeptides bind specifically to molecules on the erythrocyte surface. According to this idea, interactions among erythrocyte-specific peptides and other molecules are required to completely suppress the response to the whole erythrocyte. Alternatively, binding of a single polypeptide to a unique determinant on a foreign erythrocyte may preempt the primary antibody response to the entire cell. To distinguish between these alternatives, and to gain insight into the structural basis of specific immunologic suppression, we have examined the biologic activity of biosynthetically labeled molecules that have been purified to homogeneity. This analysis is described in the accompanying report (44).

Summary

We have generated continuously propagatable T lymphocyte clones to study antigen-specific T cell functions. All Ly-2$^+$ clones mediate suppressive activity and secrete a characteristic pattern of polypeptides that differs from Ly-2$^-$ T cell clones. Cells of one clone, Cl.Ly23/4, specifically bind glycophorin from sheep erythrocytes (SRBC). After incubation with [^{35}S]methionine, supernate material from this clone also contains biosynthetically labeled 70,000-mol wt proteins that specifically bind to SRBC and this binding is inhibited by glycophorin from sheep but not other erythrocytes. These antigen-binding 70,000-mol wt peptides specifically and completely suppress primary anti-SRBC responses generated by mixtures of primed Ly-1$^+$2$^-$ cells and B cells. Suppression by these antigen-binding peptides reflects direct inhibition of T-helper activity.

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