ANTIGEN-SPECIFIC T LYMPHOCYTE CLONES

II. Purification and Biological Characterization of an Antigen-specific Suppressive Protein Synthesized by Cloned T Cells*

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Antigen-specific T cell function is not understood in molecular terms. There is evidence that inducer T lymphocytes (Ly-1 cells) recognize antigen in association with products encoded by the I region of the major histocompatibility complex (1–3). By contrast, suppressive (Ly-2$^+$) lymphocytes and material extracted or derived from these cells can bind free antigen (4). It is likely that a portion of cell-free material responsible for antigen binding by T-suppressor (Ts)[1] cells is closely related to the cells' membrane-bound receptors (5), as is the case for B cells (6).

Studies of extracts or supernates of Ts cells have indicated two main types of antigen-specific moieties. One is likely to be a single chain of ~70,000 mol wt that does not possess I-region or immunoglobulin determinants (5, 7, 8), whereas a second group of smaller proteins bears I-region determinants (9–13). Both types of factors may bear determinants carried by the variable portion of Ig heavy chains (5, 9, 13, 14).

In the previous report, we have analyzed the properties of a cloned T cell population that expresses the surface phenotype and function associated with Ts in heterogeneous lymphoid populations (15). These cloned cells synthesized antigen-specific molecules (70,000 mol wt) that specifically preempted the antibody response to sheep erythrocytes. This analysis was based on antigen-binding properties of supernatant peptides and did not define the biologic properties of a distinct protein responsible for specific immunologic suppression. Although these data indicated that partially purified peptides completely inhibited the in vitro primary response to a complex antigen (15), this suppression might reflect the combined biologic activities of many different 70-mol wt polypeptides or polypeptides associated with 70-mol wt material by noncovalent interactions. To obtain definitive information on the structural basis of antigen-specific suppression by T cells, we have purified the relevant molecule to virtual homogeneity and analyzed the biologic activity of this purified protein.

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[1] *Abbreviations used in this paper:* BRBC, burro erythrocytes; BSA, bovine serum albumin; FCS, fetal calf serum; HRBC, horse erythrocytes; IEF, isoelectrofocusing; mc, monoclonal; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; PFC, plaque-forming cell; pI, isoelectric point; PVC, polyvinylchloride; SDS, sodium dodecyl sulfate; SRBC, sheep erythrocytes; TCA, trichloroacetic acid; Th, helper T lymphocytes; Ts, suppressor T lymphocytes.

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Materials and Methods

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

Antisera. The production and use of Lyt-1.2, Lyt-2.2, and Ly-5.1 antisera have been described in the accompanying paper (15).

Antigens and Immunization. Sheep erythrocytes (SRBC), horse erythrocytes (HRBC), and burro erythrocytes (BRBC) were purchased from the 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.

Preparation of Lymphoid Cell Populations and Bioassay Used for Protein Purification. Ly-1^+2^- cells and B cells were purified as described previously (15). Preparation of [^{35}S]methionine-labeled supernate and assessment of antigen-specific binding and suppressive activity were performed as described previously (15).

Purification of Antigen-specific Suppressor Polypeptides

All procedures were carried out at 0–4°C unless stated otherwise.

STEP 1: CONCENTRATION. After centrifugation (100,000 *g* for 30 min), [^{35}S]methionine-labeled supernate from 50×10^6 cells was concentrated 10- to 20 fold and dialyzed against Sephacryl S-200 equilibrating buffer (see below).

STEP 2: SEPHACRYL S-200 COLUMN CHROMATOGRAPHY. Concentrated supernatant material was applied to Sephacryl S-200 columns (100×2 cm) that had been equilibrated with PBS containing 2.5% glycerol, 100 mM KCl and, 0.5 mM phenylmethylsulfonylfluoride. 2-ml fractions were collected (10 ml/h flow rate) and aliquots from each fraction were tested for either biologic activity or trichloroacetic acid (TCA)-precipitable radioactivity. Columns were equilibrated with the following standards: IgG (150,000 mol wt), albumin 68,000 mol wt), ovalbumin (45,000 mol wt), soybean trypsin inhibitor (20,000 mol wt), and lysozyme (13,000 mol wt).

STEP 3: DEAE-CELLULOSE ION-EXCHANGE CHROMATOGRAPHY. Fractions containing biologic activity after Sephacryl S-200 separation were pooled and dialyzed overnight against 10 mM sodium phosphate (pH 7.2) and 2.5% glycerol. A small (6×2 cm) DEAE-cellulose column was extensively washed with the same buffer after the application of the sample to remove nonadherent protein. A 30-ml gradient, 10–300 mM sodium phosphate, was then applied to the column and remaining bound protein was eluted by increasing the salt concentration to 500 mM.

STEP 4: FLAT-BED ISOELECTROFOCUSING. This was performed in horizontal layers of Sephadex G-75 using an LKB system (LKB Instruments, Inc., Rockville, Md.) (16). After dialysis (against distilled water) of active material eluted from DEAE-cellulose, 76 ml of material was added to a solution containing 4 ml of Ampholytes (pH 3.5–8) and 4 gm Ultrodex (both from LKB Instruments, Inc.) before application to a gel tray. The tray was transferred to cooling plates (10°C) and subjected to electrophoresis for 16 h at 640 V constant voltage. After electrophoresis, the gel was sliced into 30 portions, the pH of each slice was measured, and each was passed through Sephadex G-25 columns (9×1 cm) before protein was eluted with 4 ml phosphate-buffered saline (PBS) containing 0.02% ovalbumin as carrier.

Preparation of Immunoadsorbents. The IgG fraction of different antisera was obtained by ammonium sulfate precipitation and DEAE-cellulose chromatography; different glycophorin proteins were conjugated to Sepharose as described previously (17).

Detection of Ly-1 and Ly-2 Determinants on Suppressor Polypeptides. The presence of Ly-1 or Ly-2 determinants on suppressor polypeptides was tested using a radioimmunoassay as described (18). Briefly, 100 μl of suppressive polypeptides in PBS were incubated overnight in polyvinyl plates (Cooke Engineering Co., Alexandria, Va.); PBS 1% bovine serum albumin (BSA) was used instead of agammaglobulinemic horse serum to reduce nonspecific binding of antibodies. Serial dilutions of rat monoclonal (mc)-anti-Ly-1 and anti-Ly-2 were added for 1 h at room temperature before washing and addition of ^{125}I-goat anti-rat IgG (40,000 cpm/well).

Analytical Isoelectrofocusing (IEF) in Polyacrylamide Gels. The isoelectric point (pI) of suppressor molecules was determined by IEF in slab polyacrylamide gels. Gels containing 7.5% acrylamide, 0.4% bis-acrylamide, 4% Ampholines (LKB, Instruments, Inc.) pH 3.5–8, 8 M urea and 2%

Nonidet P-40 (NP-40) were polymerized with ammonium persulfate and TEMED. Supernatant material was diluted 5- to 10-fold in a solution containing 8 M urea and 2% NP-40. The lower chamber and upper chamber of the gel contained buffers of 20 mM NaOH and 10 mM H_3PO_4, respectively. The gels were subjected to electrophoresis at 400 V constant voltage for 16 h before detection of radioactive proteins by densitometry scanning of Radioautographs after fluorographic treatment as described (19). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed as described (15).

Results

Biochemical Characterization of an Antigen-specific Ts Molecule

SEPHACRYL S-200 COLUMN CHROMATOGRAPHY OF Cl.Ly23/4 SUPERNATES (FIG. 1). Supernates of Cl.Ly23/4 contain peptides that bind specifically to determinants on SRBC and specifically suppress the response to this antigen (15). To isolate the molecule(s) responsible for these activities, biosynthetically labeled supernates were successively fractionated according to size, mobility on ion-exchange columns, and charge. After a 4-h [^{35}S]methionine pulse (15), supernates of Cl.Ly23/4 cultures were fractionated by Sephacryl S-200 chromatography under nonreducing conditions in the presence of protease inhibitors (Materials and Methods). Most of the internally labeled proteins had an apparent 20,000–100,000 mol wt (Fig. 1 A). Two fractions corresponding to apparent mol wt of 25,000 and 60,000–90,000 bound SRBC but not BRBC (Fig. 1 B). Each fraction was also tested for SRBC-specific suppressive activity (Fig. 1 C). As previously observed (15), a fraction having an ~70,000–90,000 mol wt inhibited helper activity after incubation of Ly-1 cells for 24 h. After longer periods of incubation (72 h), additional suppressive activity was detected in fractions having a 45,000 and 100,000–150,000 mol wt. Incubation of the 70,000–90,000-mol wt fraction with SRBC but not BRBC eliminated subsequent anti-SRBC suppressive activity (Fig. 1 D). In contrast, incubation of the 45,000 or 90,000–150,000-mol wt fractions with SRBC (or BRBC) had no effect on subsequent suppressive activity (Fig. 1 D).

The above data suggest that supernatant material of Cl.Ly23/4 contains two major types of suppressive activity: one is antigen-specific and has a mean 70,000–90,000 mol wt and a second is nonspecific and is apparent in two fractions having ≃45,000 and 100,000–150,000 mol wt.

DEAE-CELLULOSE ION-EXCHANGE CHROMATOGRAPHY (FIG. 2). Fractions corresponding to the 60,000–90,000-mol wt region after Sephacryl S-200 chromatography were further separated by ion-exchange chromatography. Most of the labeled protein was retained on DEAE-cellulose columns equilibrated with 10 mM sodium phosphate (Fig. 2). Elution of the column material with a salt gradient (10–300 mM sodium phosphate) demonstrated a major peak of radioactive protein (100–200 mM). SRBC-specific suppressive activity (after incubation of Ly-1 cells for either 24 or 72 h), eluted at the edge of this peak (170–200 mM) (Fig. 2).

FLAT-BED ISOELECTROFOCUSING (FIG. 3). The active fractions (170–200 mM) after elution on DEAE-cellulose were pooled and separated according to charge in Sephadex G-75 layers, using a pH gradient of 3.5–8. After focusing the gel, proteins were eluted from each of 30 fractions. Peaks of radioactivity that resolved at a pI 4.9–5.1, 5.6, and 7.6 were detected in three separate experiments. An additional peak of radioactivity, focusing at pI 5.3, was observed in one experiment (Fig. 3 A).

After removal of Ampholytes by Sephadex G-25 filtration, different fractions were

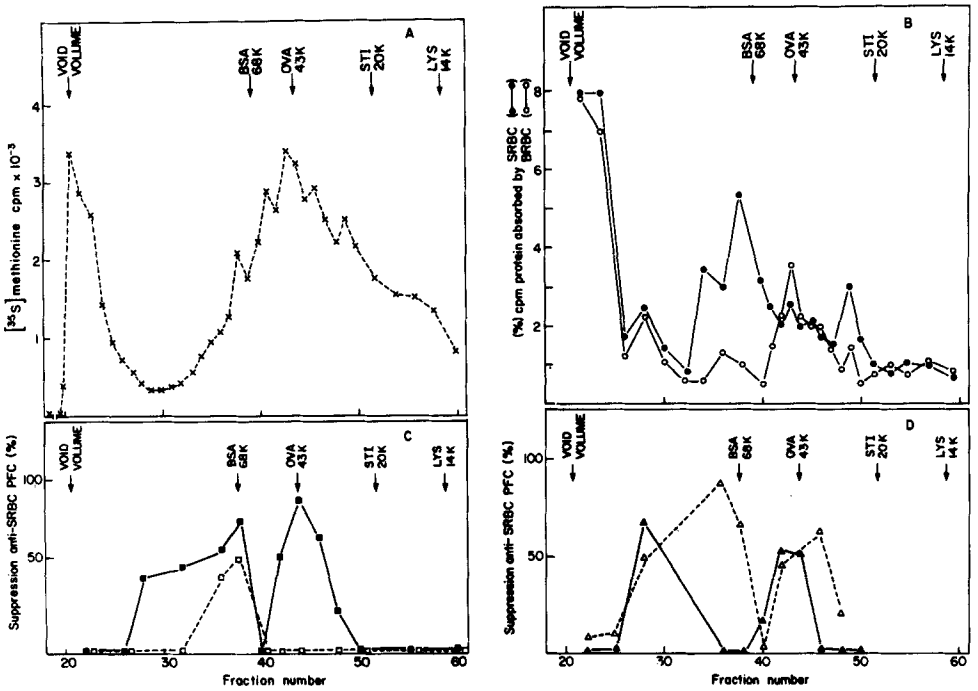

FIG. 1. Sephacryl S-200 chromatography of Cl.Ly23/4. 4-h [^{35}S]methionine-pulsed supernates from Cl.Ly23/4 cultures in serum-free media (15) were fractionated in Sephacryl S-200 columns (Materials and Methods). The fractions were tested for: (A) TCA-precipitable radioactivity (×). (B) Binding to 10^7 SRBC (●) or 10^7 BRBC (○). (C) Effects on Th activity of SRBC-immune Ly-1 cells. 10^5 Ly-1 cells were preincubated with different fractions (final dilution: 1:1,000) and 10^6 SRBC for 24 h (□) or 72 h (■). Ly-1 cells were washed twice with PBS-2% FCS before mixing with 10^6 nonimmune B cells and 10^6 SRBC. Anti-SRBC PFC were enumerated 4 d later. PFC responses induced by Ly-1 cells that had been preincubated for 24 h or 72 h with Sephacryl buffer were 2,080 ± 210 and 1,530 ± 120, respectively. (D) Effect on Th activity of SRBC-immune Ly-1 cells after preincubation of supernatant fractions with SRBC (▲) or BRBC (△). Each fraction was incubated with Ly-1 cells for 72 h before testing for Th activity. OVA, ovalbumin; STI, soybean trypsin inhibitor; LYS, lysozyme.; K, 1,000 mol wt.

tested for specific suppressive activity. The pI 4.9–5.1 fraction suppressed the PFC response to SRBC but not BRBC; the two other major fractions (pI 5.6 and 7.6) suppressed the response to both erythrocytes (Fig. 3 B). Virtually all the counts per minute of protein present in the pI 4.9–5.1 fractions bound to SRBC, whereas <5% bound to BRBC. Specific binding activity was not detected in other fractions (Fig. 3 C). It is likely that the pI 5.6 and 7.6 molecules represent degradation products of the pI 4.9–5.1 species because after freezing and thawing the 4.9–5.1 fraction, a substantial portion of the counts per minute resolves at 5.6 and 7.6 upon subsequent IEF (data not shown).

Analysis of the Purity of the Suppressor Factor Molecule (Fig. 4). These data indicate that proteins migrating at pI of 4.9–5.1 in IEF gels express antigen-specific binding and suppression. This internally labeled polypeptide might be homogeneous because nearly 100% bound to erythrocytes from sheep but not other species. To determine whether the pI 4.9–5.1 fraction indeed represented a single polypeptide, it was concentrated and applied to SDS-12.5% polyacrylamide gels. Densitometric scanning

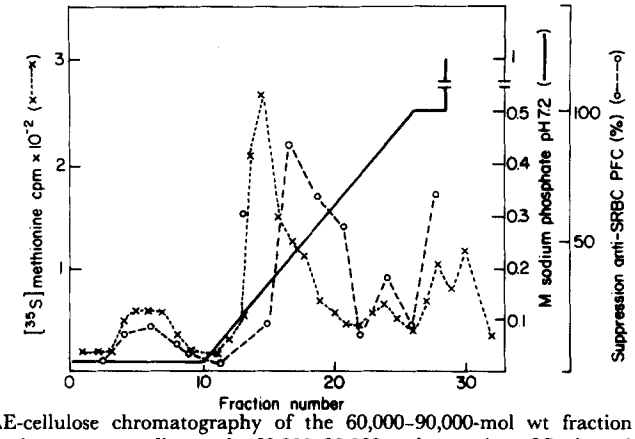

FIG. 2. DEAE-cellulose chromatography of the 60,000–90,000-mol wt fraction of Cl.Ly23/4 supernate. Fractions corresponding to the 60,000–90,000-mol wt region of Sephacryl S-200 columns (Fig. 1) were fractionated in DEAE-cellulose columns (Materials and Methods). Each fractions was tested for: (*a*) [^{35}S]methionine radioactivity (×) and (*b*) effect on Th activity of 10^6 Ly-1 cells (○) after incubation of Ly-1 cells with each fraction (dilution: 1:500). Fractions that suppressed Ly-1 cell induction of anti-SRBC PFC responses were also tested for ability to inhibit Ly-1 cell induction of anti-BRBC responses. No inhibition was detected.

of the radioautograph (Fig. 4 A) showed one narrow peak running slightly slower than BSA with an apparent 70,000 mol wt. Computation of the area under this peak indicates that 95% of the internally labeled pI 4.9–5.1 protein resolved at 70,000 mol wt. Because IEF on polyacrylamide gel can distinguish proteins of identical molecular weight but different charge, the same material was also focused on 7% acrylamide gels containing a pH gradient of 3.5–8.0. Densitometric scanning of these gels showed two very closely related peaks having an approximate pI of 4.9 and 5.0, respectively; each peak represented ~50% of the total internally labeled protein.

Specific Suppression of Anti-SRBC Responses by a 70,000-mol wt Protein (Table I). The 70,000-mol wt protein obtained from preparative IEF was tested for suppressive activity. Incubation of Ly-1 cells from donors immunized with SRBC with the 70,000-mol wt protein and SRBC eliminated subsequent anti-SRBC T helper (Th) activity (Fig. 3 C and Table I). Incubation of Ly-1 cells from HRBC or BRBC donors with the 70,000-mol wt protein and HRBC or BRBC, respectively, had no effect on subsequent anti-HRBC or BRBC Th activity.

Immunochemical Characterization of the 70,000-mol wt Protein (Table II). 1,000 cpm of internally labeled protein was mixed with 0.1 ml Sepharose beads coupled to the IgG fractions of different antisera. After incubation for 2 h at 4°C, the columns were washed with PBS and the retained protein was eluted with Sorensen's low pH buffer (glycine-HCl, pH 2.4). Because virtually all internally labeled protein (>95%) or none (<5%) were bound, the results of these experiments are recorded as positive or negative. Antisera directed against Ig and Ia antigens did not retain the 70,000-mol wt molecule. In contrast, columns coated with lentil lectin or glycophorin from SRBC—but not from human erythrocytes or HRBC—retained the 70,000-mol wt molecule. Parallel testing of suppressive activity confirmed these results: passage through columns that retained virtually all counts per minute resulted in complete loss of biologic activity in the passed material whereas passage through columns that did not retain counts per minute did not affect subsequent suppressive activity (which

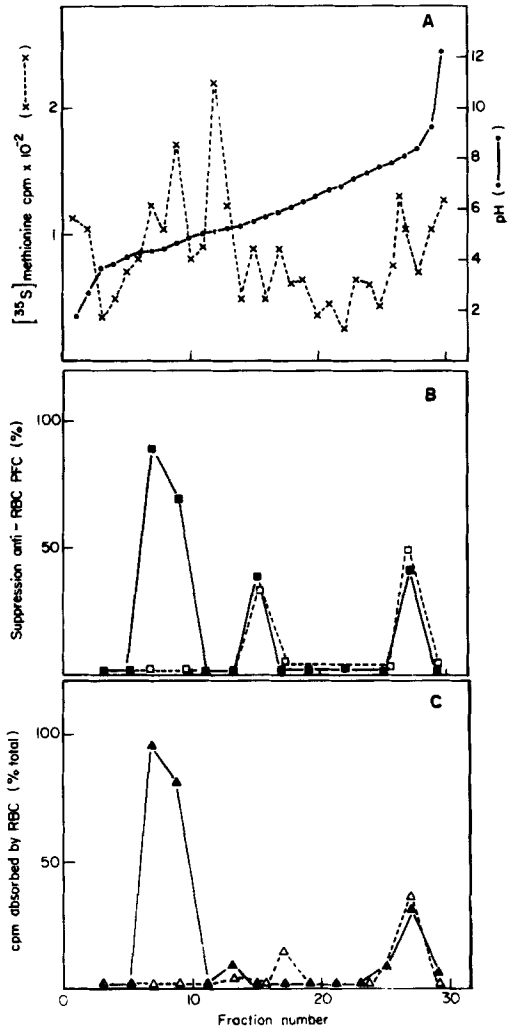

FIG. 3. IEF of DEAE-cellulose-purified Cl.Ly23/4-specific suppressive activity. Active fractions after DEAE-cellulose column fractionation (Fig. 2) were separated by charge on flat-bed IEF gels and tested for: (A) [^{35}S]methionine radioactivity (×) over the indicated pH gradient (○). (B) The effect of each fraction (final dilution 1:100) on helper activity of 10^5 SRBC-immune Ly-1 cells (■) or BRBC-immune Ly-1 cells (□) after incubation for 18 h and 10^6 homologous erythrocytes is shown. (C) Binding to 5×10^6 SRBC (▲) or BRBC (△).

was recovered in the first PBS wash). Moreover, low pH elution of bound material from antigen-coated columns or methyl mannoside elution from lectin-coated columns resulted in complete recovery of suppressive activity.

To determine whether the Ts molecule carried Ly-1 or Ly-2 determinants, 100 μl of concentrated 70,000-mol wt protein was incubated for 1 h on polyvinylchloride (PVC) dishes before addition of rat monoclonal anti-Ly-1 or anti-Ly-2 antibody and a developing ^{125}I-goat anti-rat Ig reagent. No counts above background were detected.

Moreover, addition of mc-anti-Ly-1 or mc-anti-Ly-2 antibodies did not affect suppression of Ly-1 cells by the 70,000-mol wt glycoprotein in SRBC-containing cultures (data not shown).

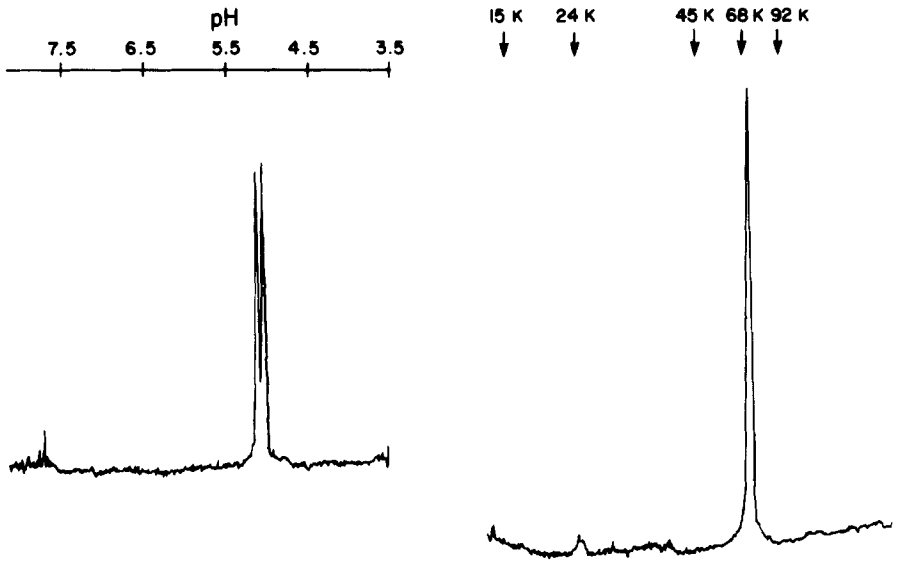

FIG. 4. Analysis of the purity of pI 4.9–5.1 fraction of Cl.Ly23/4 supernate. IEF pI 4.9–5.1 fraction (Fig. 3) was concentrated and analyzed by (12.5%) SDS-polyacrylamide gel electrophoresis (right) or IEF in polyacrylamide gels (left). Densitometric scans of the radioautographs are shown. K, 1,000 mol wt.

TABLE I

Specific Inhibition of Inducer T Cell Activity in the Presence of Antigen by the 70,000-mol wt Protein

Donors of Ly-1 cells	Incubation of Ly-1 cells for 24 h with	Test for Th activity; incubation for 96 h with	Anti-erythrocyte PFC/culture	Suppression
				%
SRBC-immune	SRBC	B cells + SRBC	654 ± 33‡	—
SRBC-immune	SRBC + SF 70K*	B cells + SRBC	54 ± 38‡	92
SRBC-immune	—	B cells + SRBC	650 ± 51‡	—
SRBC-immune	SF 70K	B cells + SRBC	459 ± 40‡	29
BRBC-immune	BRBC	B cells + BRBC	850 ± 85§	—
BRBC-immune	BRBC + SF 70K	B cells + BRBC	1,000 ± 100§	0
HRBC-immune	HRBC	B cells + HRBC	785 ± 65‖	—
HRBC-immune	HRBC + SF 70K	B cells + HRBC	695 ± 95‖	11

Ly-1^+2^- cells (10^5) from different erythrocyte-immune donors were incubated for 24 h alone or with 50 pg of purified 70,000-mol wt protein in the presence or absence of 10^6 homologous erythrocytes before testing for helper activity (see Fig. 1).

* SF 70K, IEF-purified 70,000-mol wt suppressor protein.

‡ Anti-SRBC PFC response.

§ Anti-BRBC PFC response.

‖ Anti-HRBC PFC response.

Ly-1^+2^- Helper Cells Are Targets of the 70,000-mol wt Glycoprotein (Figs. 5–7). We have shown earlier (15) that antigen-specific molecule(s) from Cl.Ly23/4 supernates inhibited anti-SRBC production in cultures containing Ly-1^+2^- helper cells and B cells by inactivating helper cells (15). Analysis of the cellular target of the purified

TABLE II

Immunochemical Characteristics of Purified Suppressive Polypeptide

	Immunoadsorbent	Counts per minute binding	Suppressive activity
A	Lentil lectin Sepharose	+*	+
	Rabbit anti-mouse Ig Sepharose	−	−
	Anti-I-J^b Sepharose	−	−
	Anti-Ia^b Sepharose	−	ND‡
	Sheep glycophorin Sepharose	+	+
	Human glycophorin Sepharose	−	−
	Horse glycophorin Sepharose	−	−
B	Rat anti-Ly-1	−	ND
	Rat anti-Ly-2	−	ND

In (A), 1,000 cpm of purified, internally labeled 70,000-mol wt protein (0.1 ml) was incubated with different immunoadsorbents coupled to Sepharose (0.1 ml) for 2 h at 4°C. Sepharose beads were then washed with 2 ml PBS and protein was eluted with 2.5 M potassium thiocyanate. Both passed and eluted fractions were concentrated and tested for radioactivity and suppressive activity. In (B), 1,000 cpm of purified 70,000 mol wt protein was coated to PVC plates and solid-phase radioimmunoassay was performed as described in Materials and Methods.

* (+), retention of counts per minute or suppressive activity; (−) no retention of counts per minute or suppressive activity.

‡ Not done.

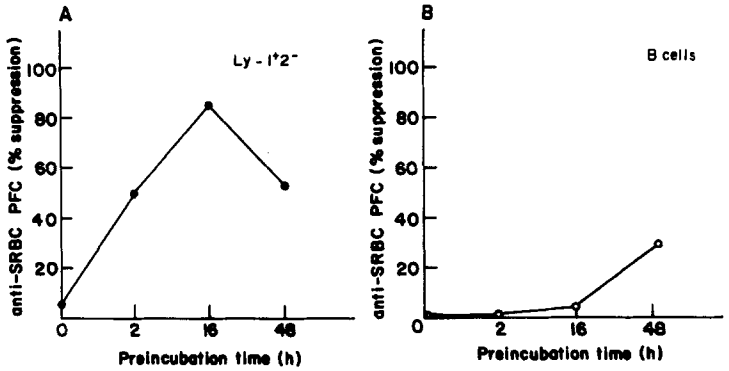

FIG. 5. Ly-1$^+$2$^-$ helper cells are targets of the 70,000-mol wt suppressor polypeptide (A) SRBC-immune Ly-1 cells (10^5) were preincubated with 50 pg of purified 70,000-mol wt molecules for the indicated periods of time in the presence of 10^6 SRBC before testing for helper activity (Fig. 1). (B) Nonimmune B cells (10^6) were similarly preincubated with 50 pg of purified 70,000-mol wt molecules and 10^6 SRBC before testing for PFC activity after mixing with 10^5 SRBC-immune Ly-1 cells and 10^6 SRBC for 4 d.

70,000-mol wt molecule confirmed and extended these studies. Incubation (for 16 h) of Ly-1 helper cells from donors immunized to SRBC 1 wk previously resulted in >80% loss of helper activity (Fig. 5 A). Incubation of Ly-1 cells and the 70,000-mol wt glycoprotein for as short a time as 2 h resulted in a 50% decrease in helper activity. B cells were also incubated with the 70,000-mol wt molecule; little or no inhibition of antibody-forming activity resulted after a 2–48-h incubation (Fig. 5 B). We tested the possibility that a portion of suppression resulted from carry-over of bound 70,000-mol

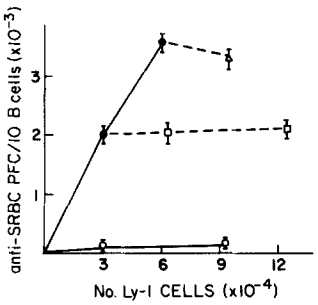

FIG. 6. Effect of suppressed Ly-1$^+$2$^-$ cells on the helper activity of fresh Ly-1$^+$2$^-$ helper cells. After incubation of SRBC-immune Ly-1 cells with 50 pg 70,000-mol wt protein and 10^6 SRBC for 24 h, no helper activity is observed (○). After incubation of SRBC-immune Ly-1 cells with buffer control, substantial helper activity was detected in this buffer control cell population (●). The anti-SRBC PFC responses induced by mixtures of 3×10^4 or 9×10^4 suppressed Ly-1 cells and 3×10^4 control Ly-1 cells (□) or 3×10^4 suppressed Ly-1 cells and 6×10^4 control Ly-1 cells (△) are shown.

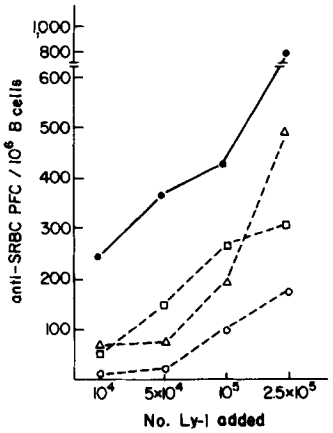

FIG. 7. Effect of different concentrations of the 70,000-mol wt protein on helper activity of Ly-1 cells. Ly-1 cells from SRBC-immune donors (10^6) were incubated for 18 h with 10^6 SRBC and 1,000 (○), 300 (△), or 100 (□) pg of 70,000-mol wt protein or control buffer (●). Anti-SRBC helper activity was tested at four different concentrations of Ly-1 cells as described in Materials and Methods.

wt protein on cells after preincubation with the suppressive molecule: Ly-1 cells that had been incubated with the suppressor molecule were mixed with Ly-1 cells that had been incubated under identical conditions in the absence of 70,000-mol wt protein. Th activity of control Ly-1 cells was not affected by the addition of suppressed Ly-1 cells that had been incubated with the 70,000-mol wt protein (Fig. 6).

Because the slopes of the inhibition curves obtained after incubation of Ly-1 cells with three different concentrations of 70,000-mol wt protein were similar, (1,000–100 pg/ml), it is possible to quantitate the potency of the material: ~10 pg of the 70,000-mol wt protein inhibited 50% of the anti-SRBC helper activity of 10^5 Ly-1 cells from SRBC-immune donors. These data also indicate the stoichiometric nature of the suppressive reaction (Fig. 7).

The above results demonstrate that the Ly-1 cells incubated with the 70,000-mol wt glycoprotein no longer provide significant Th activity to B cells, as judged from

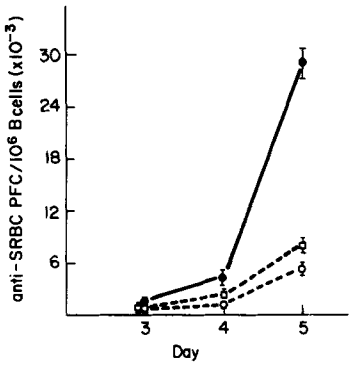

FIG. 8. Kinetics of generation of anti-SRBC PFC. Ly-1 helper cells (10^5) from SRBC-immune donors were incubated with 40 pg (○) or 20 pg (□) of the 70,000-mol wt molecule or with buffer control (●) and 10^6 SRBC for 24 h before testing for helper activity. Anti-SRBC PFC were enumerated at days 3, 4, and 5 after initiation of the anti-SRBC in vitro PFC response.

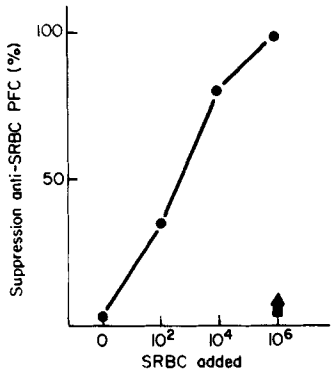

FIG. 9. Role of antigen in suppression by the 70,000-mol wt protein. SRBC-immune Ly-1 helper cells (10^5) were preincubated with 100 pg of 70,000-mol wt protein and different concentrations of SRBC (●), HRBC (▲), or BRBC (■) for 24 h, before testing for helper activity.

the anti-SRBC PFC response measured after 5 d of cell culture. We tested the possibility that the reduction in PFC formation measured at day 5 might reflect an accelerated response that generated maximal levels of PFC early after initiation of cell culture. Anti-SRBC PFC activity in these cultures is first detectable on day 3 and increases exponentially for the next 48 h (Fig. 8). After preincubation with the 70,000-mol wt glycoprotein, Ly-1 cells were unable to activate significant anti-SRBC PFC either early (day 3) or later (day 4 or 5) in the response (Fig. 8).

The Suppressor Molecule Requires the Correct Antigen to Suppress (Fig. 9). Previous studies of partially purified suppressive material indicated that suppressive activity was dependent on the presence of antigen. To quantitate this, 10^5 Ly-1 cells were incubated for 18 h with graded amounts of erythrocytes, washed and tested for SRBC-specific helper activity. Suppressive activity of 100 pg of the 70,000-mol wt protein increased in direct proportion to the numbers of SRBC, until an optimal concentration of 10^6 erythrocytes was reached. No suppression was observed in the absence of SRBC, nor with 10^6 BRBC or HRBC, confirming the fine specificity of the 70,000-mol wt molecule.

Discussion

In the accompanying paper, we have analyzed a cloned population of Ts cells. Cells of this clone bound specifically to free antigen and produced antigen-specific peptides having an apparent 70,000 mol wt. However, this analysis was based on antigen-binding properties of supernatant peptides and did not define the biologic properties of a distinct protein responsible for specific immunologic suppression. Although these data indicated that partially purified peptides completely inhibited the in vitro primary response to a complex antigen (15), this suppression might reflect the combined biologic activities of many different 70,000-mol wt polypeptides or polypeptides associated with 70,000-mol wt material by noncovalent interactions. These considerations prompted us to purify the biologically active material to homogeneity by sequential separation of molecules that mediate specific suppressive activity. Affinity chromatography, using antigen-coated columns, was not used because much of the 70,000-mol wt material is degraded on these columns, resulting in reduced recovery of protein capable of specific suppression. We therefore used classical biochemical procedures that did not affect the structure of Ts molecules and allowed insight into their biochemical properties.

In agreement with results obtained using antigen-binding (15), the use of antigen-specific suppression as a bioassay revealed a single 70,000 mol wt-molecule that specifically suppressed anti-SRBC Th activity and specifically bound to antigen. A 24,000-mol wt moiety was also routinely detected that bound specifically to SRBC but had no suppressive activity (Fig. 1 B). A second 45,000-mol wt peptide was also detected which suppressed the plaque-forming cell (PFC) response to all erythrocytes tested (Fig. 1 C). Suppression by this material was less efficient than that mediated by the 70,000-mol wt protein, and required longer periods of incubation with Th cells. The studies reported here do not bear on the relationship of the 23,000- and 45,000-mol wt peptides to the 70,000-mol wt glycoprotein. However, more recent analyses of the 70,000-mol wt molecule suggests that (*a*) 45,000- and 24,000-mol wt peptides are the principal degradation products of the 70,000-mol wt molecule and (*b*) the 45,000-mol wt peptide suppresses nonspecifically and does not bind antigen, whereas the 24,000-mol wt peptide retains antigen-binding activity but does not suppress (M. Fresno, L. McVay-Boudreau, and H. Cantor, manuscript in preparation).

Occasionally, suppressive activity was also detected in fractions having a 110,000–150,000 mol wt. Possibly, higher molecular weight activity might represent two noncovalently linked 70,000-mol wt chains; indeed, associations of this type have been reported in studies of antigen-binding material from T cells (5, 20). However, analysis of the purified 70,000-mol wt protein has not revealed a tendency to form dimers.

Because antigen-specific binding and antigen-specific suppression was confined to the 70,000–90,000-mol wt fraction after Sephacryl chromatography, this material was selected for further purification by ion-exchange and IEF gels. These separation procedures resulted in (*a*) isolation of a glycoprotein that was 95–100% homogeneous, according to SDS-polyacrylamide gels (Fig. 4) and (*b*) a recovery of 5–10% of the total 70,000-mol wt antigen-binding material originally present in unfractionated supernate.

After purification, the Ts glycoprotein migrated at 75,000 mol wt in Sephacryl S-200, and at 70,000 mol wt in SDS-polyacrylamide gel electrophoresis, using either reducing or nonreducing conditions. Because the entire purification procedure was

carried out using nonreducing conditions, it is very likely that the purified suppressor molecule is composed of a single chain. We have been unable to detect a second chain that is covalently or noncovalently associated with the 70,000-mol wt glycoprotein that contributes to specific suppressive activity. Taussig et al. (21) have reported that a SRBC-binding factor present in hybridoma supernates might be composed of two noncovalently linked chains of different sizes. These authors suggest that an association between two peptide chains is required for secretion, because only the heavier chain was detected in cell lysates. The Ts molecule described here is a single chain. If it is associated in nature with other peptides, this association is not required for antigen-specific suppressive or binding activity.

Analytic IEF of the 70,000-mol wt glycoprotein indicated two peptides of very closely related charge, suggesting that one might represent a posttranscriptional modification (Fig. 4A). Virtually all of the internally labeled 70,000-mol wt glycoprotein bound to SRBC (or sheep glycophorin), whereas <5% bound to other erythrocytes. The genetic basis of binding by the 70,000-mol wt protein is not clear: the molecule does not express conventional immunoglobulin, Ia, or Ly-1 or Ly-2 determinants (Table II). Because several anti-I-J^b antisera failed to react with the purified 70,000-mol wt molecule, we tentatively conclude that the 70,000-mol wt glycoprotein does not display easily detectable I-J determinants.

The structural basis of T cell recognition has been the object of many studies. The approaches that have been taken involve analysis of extracts (9–12), or supernates (7, 8) of heterogeneous populations of T cells, or analysis of T cells with anti-idiotypic antisera (5, 14). Although these materials have not been isolated, some carry suppressor activity, and this activity has been attributed to molecules in the 40,000–70,000-mol wt range (5, 8, 10, 22). More recently, analyses of antigen-specific material from T cell hybrids have yielded similar results (7, 21, 23–26).

In general, two main types of suppressor factors have been implied from these studies. Analysis of extracts of T suppressor cells (9–12, 14) and more recently T cell hybrids (21, 23, 24, 26) has revealed factors that may be composed of two chains: one that binds antigen and a second that bears Ia determinants. An exception has been reported recently by Pacifico and Capra (25) who have identified a single 62,000-mol wt peptide that binds to antigen and that may also express Ia determinants. However, the functional activity of this peptide has not been established. A second type of antigen-specific material, recovered from supernates or membranes of uncloned T cells, has an ~70,000 mol wt and lacks Ia determinants (5, 7, 8, 20, 22).

These observations, taken together with functional studies, suggest two types of molecules that may play important roles in the generation and expression of Ts activity and in antigen recognition. The first includes material that may have two chains and I-region products (9–14, 21, 23, 24); these molecules are important in T cell-T cell interactions that generate Ts from Ly-2^+ precursors (10–13). The second directly mediates suppression and is composed of a single 70,000-mol wt chain, lacks I-region determinants, and likely represents a secreted form of the T cell receptor (5, 7, 8, 20, 22).

The molecule described here belongs to this latter group. Picogram amounts of this glycoprotein can specifically suppress the Th response to a foreign erythrocyte. A molecular explanation of this activity comes from the finding that the 70,000-mol wt molecule does not suppress Ly-1 Th cells in the absence of the correct erythrocyte.

Antigen is not required to stimulate Th cells and perhaps render them more sensitive to suppression because Th cells from BRBC- or HRBC-immune donors are not suppressed after incubation in vitro with the 70,000-mol wt protein and the erythrocyte used for immunization. Recent studies have indicated that Ly-1 cells can bind to radiolabeled antigen that has been processed by macrophages (27). Because the 70,000-mol wt molecule specifically binds to SRBC, antigen can form a bridge between the Ts molecule and antigen-specific Th cells. Possibly, antigen bound to Ly-1 cells may focus Ts molecules to correct Ly-1 cells, resulting in specific suppression of anti-SRBC Th activity.

Several mechanisms might account for the ability of a single glycoprotein to preempt the entire primary antibody response to a foreign erythrocyte. The explanation that we favor is based on the structural properties of the 70,000-mol wt molecule. After interaction with the antigen, the 70,000-mol wt protein breaks down into two peptides having molecular weights of 45,000 and 24,000 mol wt. The former peptide displays nonspecific suppression and does not bind to antigen; the latter does not possess detectable Ts activity but binds specifically to SRBC (M. Fresno, L. McVay-Boudreau, and H. Cantor, manuscript in preparation). These findings suggest that occupation of the antigen-binding portion of the Ts molecule may result in activation of the 45,000-mol wt portion and suppression of Ly-1 cells, regardless of their specificity for antigen.

Although suppression may result from a direct interaction between the Ts molecule and Ly-1 cells, a second possible mechanism comes from studies of antigen-recognition by Th. Ly-1^+ Th cells are thought to recognize antigen in association with Ia molecules on the surface of antigen-presenting cells (1–3). Although positively selected Ly-1 cells incubated with the 70,000-mol wt protein are >98% Ly-1^+2^- according to immunofluorescence, contamination by macrophages cannot be excluded. Possibly, Ly-1 cells may recognize complexes formed by the Ts molecule and antigen on the surface of macrophages. Suppression of Ly-1 cells may thus reflect either direct inhibition by the 70,000-mol wt molecule-antigen complex or nonspecific inhibitory molecules produced by macrophages after interaction with the 70,000-mol wt molecule-antigen complex. In the latter case, cells obtained after incubation with the 70,000-mol wt molecule and antigen might be expected to inhibit helper activity of fresh Ly-1 cells. Although this was not the case (Fig. 6), this finding does not rule out the potential role of macrophages in antigen-specific suppression: cells recovered after 18–24 h incubation in vitro may have been depleted of potentially suppressive adherent cells. Experiments aimed at pinning down the role of antigen-presenting cells in suppression and further analysis of the biologic functions of different regions of the suppressive molecule should resolve these questions.

Summary

We have generated an antigen-specific T suppressor clone that synthesizes 70,000-mol wt peptides that have antigen-specific-binding activity. Although these data also indicated that antigen-binding peptides completely inhibited the in vitro primary response to a complex antigen, suppression might reflect the combined biologic activities of many different 70-mol wt polypeptides or polypeptides associated with the 70,000-mol wt material by noncovalent interactions. The protein responsible for antigen-specific suppression was therefore purified to virtual homogeneity after se-

quential separation of internally labeled supernate peptides on Sephacryl S-200 and DEAE-cellulose columns followed by isoelectrofocusing. The resulting protein is >95% homogeneous according to sodium dodecyl sulfate-polyacrylamide electrophoresis and represents two peptides having two very close but distinguishable isoelectric point values of ~5.0.

The purified molecules are retained by columns coated with lentil lectin or antigen but not by columns coated with antisera specific for immunoglobulins, the I region of the major histocompatibility complex or Ly-1 or Ly-2 antigens. Less than 50 pg of the purified glycoprotein specifically and completely suppresses production of anti-sheep erythrocyte plaque-forming cell by mixtures of 10^6 Ly-1 cells and B cells and this is a result of inactivation of Ly-1-mediated helper function. Specific inactivation of T (Th) cells by the 70,000-mol wt molecule is rapid, specific, and requires the presence of antigen. The mechanism of specific suppression of Th function may depend upon two functionally distinct regions of the 70,000-mol wt molecule: one that binds antigen and a second that mediates suppression.

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