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Immunology

Isolation and partial characterization of an antigen-specific T-cell factor associated with the suppression of delayed type hypersensitivity

ROBERT W. ROSENSTEIN*†, JAMES H. MURRAY‡, ROBERT E. CONE*, WLODZIMIERZ PTAK§, G. MICHAEL IVERSON*†, AND RICHARD K. GERSHON*†

*Yale University School of Medicine, Department of Pathology and †Howard Hughes Medical Institute, Laboratory of Cellular Immunology, Yale University, New Haven, Connecticut 06510; and §Copernicus Medical University, Cracow, Poland

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ABSTRACT **Antigen-specific factors associated with immunosuppressive activity, released by cultured T cells from mice tolerant to the haptens trinitrophenyl, dinitrophenyl and oxazolone, were purified by hapten affinity chromatography. Their binding specificity for antigens paralleled their immunoregulatory activity. Like some immunoglobulin molecules, these factors had blocked NH_2 termini and could be bound to Fc-like receptors on macrophages. However, neither immunoglobulin constant region determinants (isotypes) nor antigens encoded by the major histocompatibility complex were detected on the suppressive factors. The purified factors occurred as 68,000-dalton proteins and noncovalently linked dimers. No associated immunoglobulin light chain molecules were detected. The factors showed a marked propensity toward degradation with major breakdown products of 45,000–50,000 and 25,000–30,000 daltons. These results suggest that these molecules are the T-cell products analogous to B-cell immunoglobulin (equivalent to heavy chains) and that they may be the antigen-specific components which act in conjunction with major histocompatibility-controlled gene products to perform antigen-specific suppression.**

Central to our understanding of the mechanism by which lymphocytes respond to antigenic signals is elucidation of the nature of the surface membrane receptors for antigen. B and T cells, as part of their function, release soluble antigen-specific molecules that are capable of effector function (1, 2). B-cell-derived soluble effector molecules are immunoglobulins whose function in the immune system is dictated by their antigen binding specificity and the nature of the constant portion of the molecule. Although constant regions of circulating immunoglobulins may differ from those of their membrane-associated counterparts, the two forms of immunoglobulins express identical variable regions which perform binding functions. A similar situation obtains for at least some T lymphocytes. Antigen recognition at the cellular level is mediated by membrane receptors, and T-cell effector function is carried out by soluble antigen-specific products. However, although both cell-bound and soluble forms of T-cell antigen-binding molecules have been shown to bear antigenic determinants associated with immunoglobulin combining sites (idiotypes) (3), the overall structure of these molecules has yet to be clarified.

As has been the case for B cells, detailed analysis of T-cell derived soluble antigen-specific effector molecules should illuminate the mechanisms by which T lymphocytes perform their effector function in the immune system. Moreover, although T cell-released soluble products may not be identical to T cell-membrane receptors for antigen, they provide a vehicle that can be used to clarify the nature of T cell-membrane receptors. In this report we describe the isolation and partial characterization of T cell suppressor factors specific for dinitrophenyl (DNP), trinitrophenyl (TNP), or oxazolone (Ox).

MATERIALS AND METHODS

Materials. Bovine gamma globulin (BGG) was purchased from Sigma; fluorodinitrobenzene and trinitrobenzenesulfonic acid were from Eastman; picryl chloride was obtained from Chemtronix (Swann Anoa, NC) and was recrystallized twice from methanol; Ox was from Gallard–Schlessinger (Carle Place, NY) (prior to use it was recrystallized from methanol/H_2O); CBA/J mice were from The Jackson Laboratory; several anti-I-J^k and anti-I (AT.H anti-AT.L) antisera were kindly donated by Donal Murphy (Yale University). The anti-I-J^k was produced by injecting B10.A(5R) spleen cells into B10.A(3R) recipients; anti-mouse isotype reagents were purchased from Litton Bionetics (Kensington, MD) and Gateway Immunoserum (St. Louis, MO). Their specificity was tested by Ouchterlony double-diffusion analysis against myeloma proteins (Litton); goat anti-mouse Ig was affinity purified by adsorption to and elution from mouse Ig-Sepharose 4B columns. Mouse Ig used for this purification was prepared by salting out pooled normal mouse serum with 18% (wt/vol) Na_2SO_4; sheep anti-rabbit Ig was obtained from Heinz Furthmayr (Yale University) and was affinity purified by adsorption to and elution from rabbit Ig-Sepharose 4B; [^{35}S]methionine, [^{3}H]leucine, and Na^{125}I were obtained from New England Nuclear. Tissue culture media used for the generation of T-cell suppressor factor (TsF) was RPMI-1640 (GIBCO) lacking the amino acid to be used for biosynthetic labeling.

General Methods. Affinity chromatography was performed with Sepharose 4B as the support matrix. Proteins were covalently attached to it by the CNBr technique of Axen *et al.* (4). For purification of TsF, haptens were covalently attached to BGG-Sepharose at pH 11 (0.2 M Na_2CO_3) by using TNBSA for TNP, fluorodinitrobenzene for DNP, and Ox. These last two were first dissolved in ethanol and added to reaction mixtures so that 10% of the reaction mixture volume was ethanol.

Gel filtration for molecular weight determinations was done

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Abbreviations: DNP, dinitrophenyl; TNP, trinitrophenyl; BGG, bovine gamma globulin; EACA, ε-aminocaproic acid; Ox, oxazolone; P_i/NaCl, phosphate-buffered saline, pH 7.4; TsF, T-cell suppressor factor; TX/P_i/NaCl, Triton X-100 in P_i/NaCl; MHC, major histocompatibility complex.

‡ Present address: New England Nuclear, Immunochemistry Section, Boston, MA 02118.

on Sephacryl S-200 (Pharmacia) with pH 7.4 phosphate-buffered saline (Pi/NaCl) as the elutant. Radioactive samples were dialyzed prior to gel-filtration. $NaDodSO_4$/polyacrylamide gel electrophoresis of ^{125}I-labeled or ^{35}S- and ^{3}H-labeled proteins was performed as described (5, 6).

Protein iodinations (TsF, sheep anti-rabbit Ig, goat anti-mouse Ig) were done by the lactoperoxidase method (6, 7).

Liquid scintillation counting was done in Aquasol (New England Nuclear) in a Beckman liquid scintillation spectrometer. ^{125}I determinations were done in a Beckman Biogamma or an LKB gamma spectrometer. The binding of TsF to haptens coupled to BGG-Sepharose was performed as follows. Solid-phase affinity matrix was washed with 0.05% Triton X-100 in P_i/NaCl and 0.2 ml was pipetted into 16 × 75 mm tubes (Falcon). To this was added 0.1 ml of radioactive TsF to be tested. The assay tubes were agitated at 25°C every 10 min for 1 hr. Each affinity matrix was pelleted at low speed in a clinical centrifuge, and 0.1 ml was removed for radioactivity determinations. In the case of ^{125}I-labeled TsF, the affinity matrix was washed four times with 0.05% TX/P_i/NaCl and bound radioactivity was determined directly.

Production of Suppressor Factors. TsF specific for TNP or DNP was generated by the method of Zembala and Asherson (8) modified to yield maximal suppression of Ig production (9). Mice received two intravenous injections of 5 mg of trinitrobenzenesulfonic acid or dinitrobenzenesulfonic acid 3 days apart. Six days after the final injection, the skin of the mice was painted with 5% picryl chloride or 0.5% dinitrofluorobenzene. One day after painting, spleen and lymph node cells from these mice (approximately 1–2 × 10^9 cells) were cultured at 1.5 × 10^7 cells per ml in serum-free RPMI-1640 medium for 48 hr. For biosynthetic labeling, the cells were cultured in leucine- or methionine-free RPMI supplemented with 10 mCi (1 Ci = 3.7 × 10^{10} becquerels) of [^{3}H]leucine or [^{35}S]methionine. After 48 hr the culture fluids were separated by centrifugation and processed for isolation of TsF. Ox-specific TsF was prepared and assayed as described (10).

TsF was purified from culture fluids by adding them (100–200 ml) to affinity matrices and incubating the mixture overnight at 4°C with stirring. The mixtures were then poured into columns and washed with five column volumes of P_i/NaCl. The columns were then eluted with 0.05 M TNP-ε-aminocaproic acid (EACA), DNP-glycine, or Ox-EACA. The eluates were then exhaustively dialyzed into Pi/NaCl. NH_2-terminal amino acid analysis of TsF preparations was done by the method of Gray and Hartley (11).

Bioassay of TsF. The ability of TsF to suppress the transfer of delayed type hypersensitivity to naive mice was the biological assay of activity (9).

Immunoassays. Three types of immunoassays were performed: direct binding, competitive binding, and soluble precipitation.

Direct binding assays consisted of coating TsF preparations onto the wells of flexible vinyl microtiter trays at a concentration of 1 μg/ml in 0.01 M borate at pH 8.5. The coating was allowed to proceed for 18 hr at 4°C. The trays were then washed with distilled water, dried in an oven at 37°C, and stored under reduced pressure in a desiccator at 4°C. Dilutions of test antisera were made in 0.05% TX/P_i/NaCl in 2-fold steps starting at 1:10. Aliquots (0.1 ml) of diluted test antisera were added to the TsF-coated wells and allowed to incubate for 18 hr at 4°C. The trays were then washed four times with 0.05% TX/P_i/NaCl and excess liquid was removed. ^{125}I-Labeled second antibody (sheep anti-rabbit or goat anti-mouse) was added to each well (50,000 cpm in 0.1 ml) and allowed to incubate for 2 hr at 25°C. The trays were washed and dried, and individual wells were removed for radioactivity measurements. Controls consisting of uncoated wells or TsF-coated wells incubated with pooled normal serum were included in the same tray.

Competitive assays were also performed on solid supports using flexible vinyl microtiter trays, except dilutions of specific antisera were used to coat the wells. A test coating of dilutions of antiserum in 0.01 M borate (pH 8.5) was first done to determine the correct dilution to be used in the competitive binding assay. Coated wells were incubated for 18 hr at 4°C with 0.1 ml of 0.05% TX/P_i/NaCl containing varying amounts of TsF and 50,000 cpm of ^{125}I-labeled antigen. The wells were then washed four times with 0.05% TX/P_i/NaCl and dried, and individual wells were removed for radioactivity determinations. Quantitative results were obtained by including wells for a standard curve on the same tray as the assay. The standard wells contained various dilutions of nonradioactive antigen in place of TsF.

Immunoassays in solution used 5–10 μl of radiolabeled (^{125}I, ^{3}H, ^{35}S) TsF added to 200 μl of 0.05% TX-Tris (50 mM Tris/10 mM EDTA/0.15 M NaCl, pH 8.0). Rabbit or mouse antiserum (10–20 μl) was then added and the mixtures were incubated at 4°C for 1 hr. Immune complexes were precipitated with formalin-fixed *Staphylococcus aureus* Cowan strain I (Calbiochem) or sheep anti-rabbit Ig antiserum. After a 10-min incubation with *S. aureus* or overnight incubation for immunoprecipitates, samples were washed twice with 0.05% TX-Tris buffer and then the radioactivity in the pellet was determined.

RESULTS

Isolation of Antigen-Specific TsF Activity by Hapten-Affinity Chromatography. Antigen-specific TsF decreased the adoptive delayed hypersensitivity response of recipients by ≈70–90%. The suppressive culture supernatants were absorbed to TNP-, DNP- or Ox-BGG coupled to Sepharose 4B. The appropriate hapten columns bound specific suppressor activity that could be eluted by hapten (Table 1). Thus, TNP-specific TsF eluted from TNP-BGG-Sepharose by TNP-EACA suppressed the transfer of a delayed hypersensitivity response of TNP-sensitized cells by 85–90%, suppressed the activity of DNP-sensi-

Table 1. Hapten-specific TsF can be eluted from affinity columns with hapten

Sample	% suppression*	
	Experiment 1	
TNP TsF eluted from TNP-BGG column with TNP EACA	85, TNP system;	0, Ox system
Ox TsF eluted from Ox BGG column with Ox-EACA	0, TNP system;	100, Ox system
	Experiment 2	
TNP TsF elute from TNP-BGG column with TNP-EACA	90, TNP system;	30, DNP system
DNP TsF eluted from DNP-BGG column with DNP-glycine	2, TNP system;	90, DNP system

All groups consisted of four animals. Positive control animals received hapten-specific immune T cells. Negative control animals received normal T cells. Experimental group animals received hapten-specific immune T cells preincubated with TsF. Positive control, 7–9 mm; negative control, 1–2 mm.

* % suppression calculated from 24-hr ear swelling (measured in mm) as follows:

$$100 \times \frac{\text{experimental} - \text{negative control}}{\text{positive control} - \text{negative control}}$$

tized cells by 30%, and had no effect on the transfer of delayed hypersensitivity by Ox-sensitized cells. The Ox-specific TsF eluate suppressed the activity of cells sensitized to Ox only. Similarly, hapten affinity-purified DNP-specific TsF suppressed DNP-sensitized cells only.

All preparations of TsF purified by hapten affinity-chromatography were also subjected to NH_2-terminal amino acid analysis by the dansyl chloride method (11). No detectable NH_2-terminal amino acid could be found, suggesting that TsF molecules have a blocked NH_2-terminus.

Binding Specificity. Biosynthetically labeled or, in some cases, ^{125}I-labeled proteins were adsorbed to hapten affinity-columns and, after washing, the columns were eluted with nonspecific or specific eluants. In general, approximately 1–2% of the total radiolabeled proteins were adsorbed by the affinity columns. The amount of ^{35}S-labeled proteins released by normal cells that could be adsorbed to the affinity columns was less than 1/10th the amount of labeled material produced by cells obtained from tolerized animals. Labeled TsF could not be eluted with the nonspecific hydrophobic hapten carbobenzoxy glycine (Fig. 1), indicating that the hydrophobic nature of the haptens tested was not responsible for their binding of the factors. Approximately 70% of the bound proteins were specifically eluted with hapten, after which the remaining proteins were eluted by Na_2CO_3 pH 11 buffer. Each of the antigen affinity-purified molecules was bound again to antigen with a high degree of specificity (Table 2). As measured by the Lowry assay, approximately 1 mg of protein could be purified from 50–100 mice.

Molecular Sizing of TsF. The molecular size of TsF was ascertained in two ways. First, [^{35}S]methionine-labeled proteins in culture fluids were fractionated by chromatography through Sephacryl S-200 equilibrated in P_i/NaCl (Fig. 2). Suppressor activity and antigen binding activity were detected only in column fractions corresponding to polypeptides with M_r 150,000–400,000. Some antigen binding activity, without suppressor activity, was detected in column fractions corresponding to polypeptides of M_r 45,000. Under reducing conditions (Fig. 3), the major molecular species detected had an apparent M_r of 65,000–72,000.

In some preparations, minor species corresponding to M_r of 45,000 or 25,000–30,000 were observed. Resolution of these polypeptides under nonreducing conditions resulted in similar patterns. That these minor species may be degradation products of larger polypeptides is suggested by the appearance in $NaDodSO_4$/polyacrylamide gel electrophoresis of lower molecular weight species when purified TsF that had been rebound and eluted from antigen columns with $NaDodSO_4$/urea or stored for 5 months was analyzed (Fig. 4).

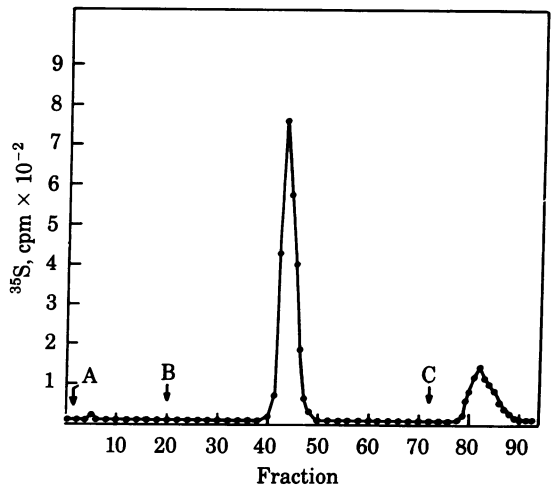

FIG. 1. Hapten elution of TsF from antigen affinity columns. ^{35}S-Labeled TNP-specific TsF was eluted from a TNP-BGG-Sepharose immunoadsorbent column with 0.05 M TNP-EACA (B) but not a nonspecific hydrophobic hapten, 0.05 M carbobenzoxyglycine (A). Only a small additional amount of ^{35}S-labeled protein was eluted with 0.2 M Na_2CO_3 at pH 11 (C).

Table 2. Binding of radiolabeled affinity-purified TsF to antigen

Exp.	Specificity of TsF*	Hapten affinity matrix†	cpm bound‡	Ratio of binding§
1	Ox	TNP-BGG	11,000	4.72
		Ox-BGG	51,900	
2	Ox	TNP-BGG	4,110	10.5
		Ox-BGG	43,100	
3	TNP	TNP-BGG	75,000	4.7
		Ox-BGG	16,000	
4	DNP	DNP-DAP	124,500	5.5
		Ox-DAP	22,810	

* TsFs were purified from culture supernatants by hapten affinity columns.
† Haptens on BGG or diaminopropyl (DAP) covalently attached to Sepharose 4B.
‡ ^{125}I-labeled TsF; label introduced after affinity purification.
§ cpm bound to homologous hapten/cpm bound to heterologous hapten.

Binding of Affinity Purified TsF to Macrophages. Previous studies (9) have shown that the suppressive activity of TsF requires the presence of macrophages and that these cells can absorb suppressive activity from culture supernatants containing TsF. Consequently, we determined if ^{125}I-labeled TsF would bind to various cell populations (Table 3). ^{125}I-Labeled TsF was bound by cell populations rich in macrophages from peritoneal exudates and not by those containing relatively few macrophages. Inclusion of 100 μg of heat-aggregated IgG with 3×10^6 macrophages inhibited the uptake of TsF by 90%; nonaggregated IgG inhibited by 62%, and fetal calf serum had no effect. Treatment of the macrophages with Pronase or trypsin abrogated the absorbtive capacity of the cells for TsF; treatment of the cells with neuraminidase had no effect.

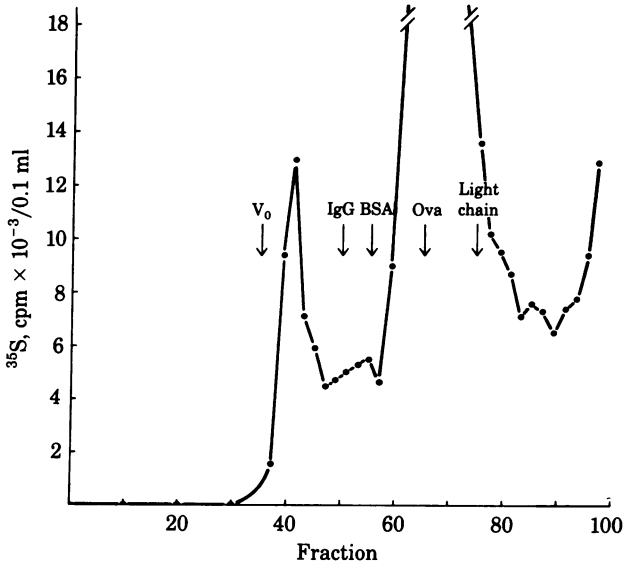

FIG. 2. Gel-filtration of TsF. ^{35}S-Labeled proteins from a TNP-specific TsF cell-free culture were separated on a 2.5 × 90 cm Sephacryl S-200 column eluted with P_i/NaCl. Only the first radioactive peak, eluting at fraction 40, had both suppressor activity and hapten-binding specificity. BSA, bovine serum albumin; Ova, ovalbumin.

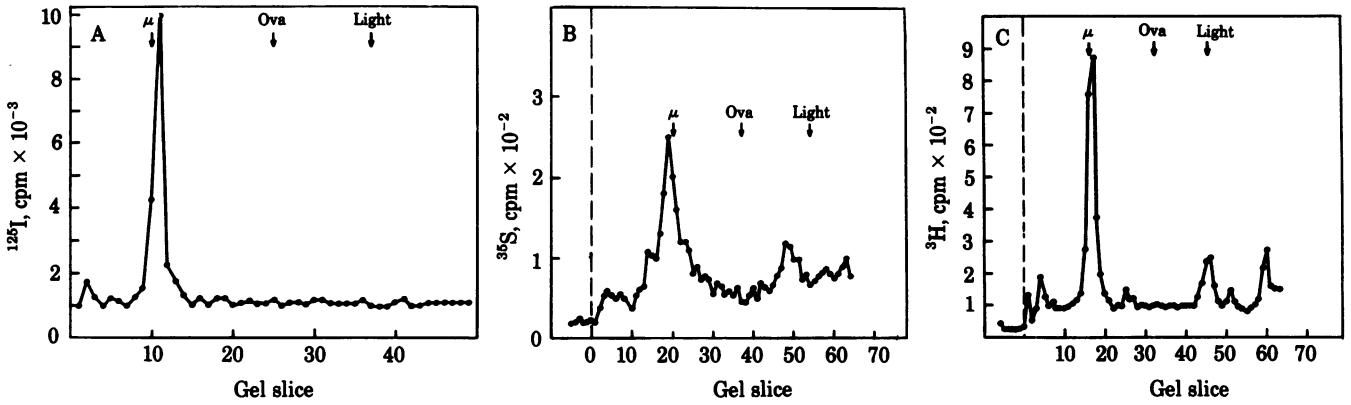

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of hapten-specific TsF. TsF were externally labeled with ^{125}I (*A*; DNP specific) or internally labeled with ^{35}S (*B*; Ox specific) or ^{3}H (*C*; TNP specific) during culture.

Serological Analysis of TsF. We explored the serological identity of TsF with a series of specific immunoassays using TsF as a competitive inhibitor, using TsF coated onto plastic microtiter wells, and using ^{125}I-labeled TsF, antiserum, and *S. aureus* protein A to precipitate any complex formed (Table 4). No immunoglobulin heavy or light chain isotypic determinants were detected, nor were murine albumin, viral protein gp70, or major histocompatibility complex (MHC) region I, or Lyt determinants. In addition, adsorption of culture fluids containing TsF to anti-Ia^k- or anti-I-J^k-conjugated Sepharose beads did not remove suppressor activity. All these immunoadsorbents were able to bind their specific ligands.

DISCUSSION

These results show that the soluble mediators of antigen-specific suppression made by T cells of mice made tolerant to reactive haptens (9) have the following chemical characteristics:

(*i*) Antigen binding and suppressor activity are associated with molecules in the size range of 70,000–150,000 daltons and, in some cases, larger weight species. The larger species are probably oligomers of noncovalently linked 65,000- to 72,000-dalton monomeric polypeptide chains as ascertained by NaDodSO$_4$/polyacrylamide gel electrophoresis. Thus, the basic antigen-specific unit of TsF is similar to immunoglobulin heavy chains in size.

(*ii*) Like immunoglobulin, TsF binds to receptors on macrophages and this binding can be inhibited by aggregated Ig, suggesting that the receptors are related to receptors for the Fc portion of Ig. This binding is crucial to the effector function (9) of TsF and is reminiscent of the cytophilic binding of Ig-like molecules derived from T-cell membranes but not from B cells (12). The failure to find Ig markers in the hapten affinity-purified proteins indicates both that the TsF has no standard in Ig markers and that the tolerizing regimen was effective in shutting off B-cell responses. Because antigen-specific T-cell proteins are labile, the presence, in NaDodSO$_4$ gels, of light chain-like molecules obtained from T-cell isolates cannot be taken as evidence for T-cell light chain without further documentation.

(*iii*) Hapten-specific TsFs do not express antigenic determinants encoded by the MHC genes. Although negative data cannot definitively rule out the presence of MHC gene products on TsF, they do rule out the presence of those MHC gene products with which our antisera have been shown to react: a T suppressor inducing cell and its cell-free mediator (13); the acceptor cell for this mediator (13); the inducer, acceptor, and effector cells in the contrasuppressor circuit and their cell-free mediators (14); and a non-T non-B cell required for a number of *in vitro* immune responses (15). Conspicuously missing from this list is the effector cell(s) of suppression and (their) cell-free product(s). In other systems we find these also to be MHC negative (13).

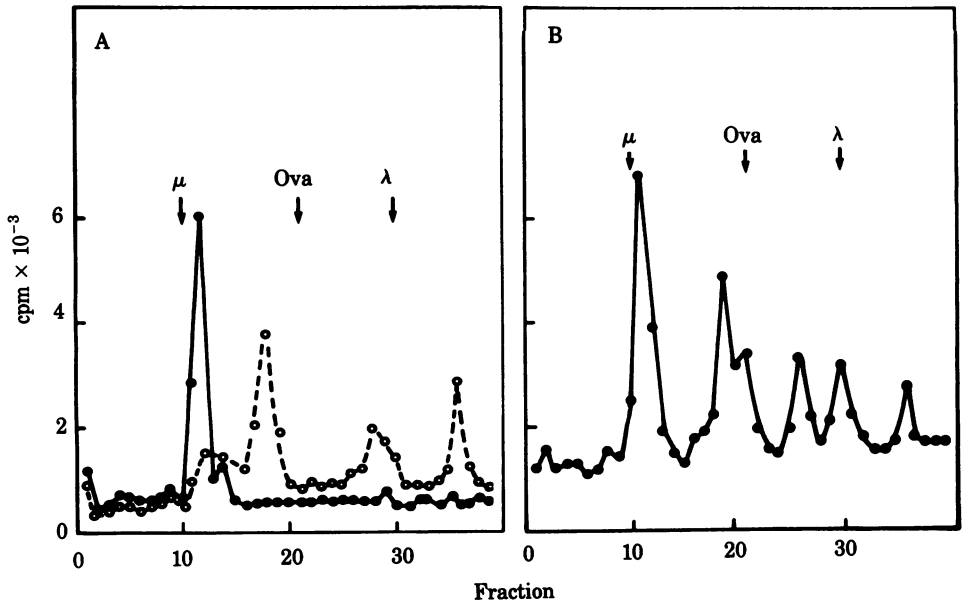

FIG. 4. Spontaneous degradation of TsF. Hapten affinity-purified DNP-specific TsF (68,000 daltons) was rebound to DNP-BGG-Sepharose and eluted with NaDodSO$_4$/urea sample buffer. ●——●, at original isolation; ○----○, after rebinding and elution. (*A*) With NaDodSO$_4$/urea sample buffer; (*B*) after 5-month storage (with several freeze-thaw cycles).

Table 3. Cytophilic binding of ^{125}I-labeled TsF (cpm bound)

Cells, no.	Macrophages*	Macrophages + aggregated Ig†	Spleen cells	Bone marrow cells	Thymocytes
3×10^6	18,900	2000	1500	500	750
30×10^6	18,100	ND	6100	2500	920

^{125}I-labeled TsF was incubated with cells in 100 μl of P_i/NaCl for 1 hr at 4°C. Cells were washed three times before assay of radioactivity.
* Peritoneal exudate cells.
† With 100 μg of heat-aggregated (62°C, 30 min) mouse IgG. The following materials (at 100 μg) competed significantly but less well for binding sites of the macrophages: nonaggregated mouse IgG (62% inhibition), normal mouse serum (52% inhibition), bovine IgG (17% inhibition). Fetal calf serum showed no inhibition. ND, not done.

In addition, Fresno *et al.* (16) found a suppressor effector clone and its cell-free product to be MHC negative. The biologically active cell-free product of that clone resembles in many ways the TsF we have described.

On the basis of these and other findings (17, 18), we suggest that suppressor T cells (or the cells with which they communicate) make two or more distinct polypeptides to effect suppression. One is the antigen-binding moiety we have described, and the other is an MHC-controlled polypeptide that imparts biological function. These two polypeptides may interact to form a molecular complex at the time they are released from the producer cell(s) or at the site where suppression is being effected. In the case of the TsF we have described, that site may be on the surface of macrophages (9). Thus, T cells may release a polypeptide that is analogous to antibody released by B cells. Functional effects in both cases require that the antigen-specific products interact with other cells or molecules. In the case of antibody, one of its important functions is dependent on its interaction with complement; in the case of the TsF we have described, function may depend on its interaction with MHC gene products. The location of a gene locus controlling the production of a complement component in the MHC (19) may not be totally fortuitous.

Table 4. Survey of serological determinants associated with CBA/J-devised TsF

Reagent used	Assay method*
Negative results	
Rabbit reagent:	
Anti-κ	RIA direct binding
λ	RIA direct binding
IgA	RIA direct binding
IgM	RIA competitive
IgE	RIA direct binding; RIA competitive
IgG	RIA direct binding; RIA competitive
Mouse albumin	RIA direct binding; precipitation
gp70	RIA direct binding; RIA competitive
Mouse reagent:	
A.TH anti-A.Tl (anti-Ia^k)	RIA direct binding; adsorption
3R anti-5R (anti-I-J^k)	RIA direct binding
Monoclonal antibodies:	
Anti-Ig5a (anti-IgD)†	RIA direct binding
Anti-LgT-1‡	RIA direct binding
Anti-LgT-2‡	RIA direct binding
Binding >3 times background	
Rabbit reagent:	
Anti-TsF	RIA direct binding; precipitation
Anti-rat T cell receptor§	RIA direct binding; precipitation

* RIA, radioimmunoassay. For adsorption, A.TH anti-A.TL-Sepharose was used as adsorbent. TsF biological activity was measured before and after adsorption.
† From Becton Dickinson.
‡ Derived from cells obtained from N. Warner in conjunction with the National Cancer Institute.
§ Gift of Hans Benz (University of Zurich).

The explanation offered above is supported by data from studies of T-cell hybridomas (20, 21) and Ly-1 T inducer cells (unpublished data) which have clearly shown that the I-J$^+$ material in T-cell factors is on a different polypeptide than the one that binds to antigen. Because some studies have failed to separate MHC products from antigen-binding material released by T cells, the MHC product may become tightly bound to the antigen-recognizing moiety [perhaps covalently as Taniguchi has suggested (20)]. Thus, the antigen-specific moiety we have described may react with another polypeptide, probably MHC positive, in order to perform its biological role. Preliminary evidence supports this expectation.

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