

PURIFICATION AND PHYSICOCHEMICAL CHARACTERIZATION OF MURINE T CELL REPLACING FACTOR (TRF)¹

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Murine T cell replacing factor (TRF) was purified from a cellfree supernatant of a T cell hybridoma (B151K12) that constitutively produces TRF. Two assay systems for TRF activity were employed: 1) induction of anti-DNP IgG PFC responses in cultures of splenic B cells from DNP-KLH-primed BALB/c mice, and 2) induction of IgM PFC in chronic B cell leukemic cells (BCL₁). The purification scheme consisted of ammonium sulfate precipitation, DEAE-cellulose chromatography, Blue-Sepharose chromatography, hydroxylapatite chromatography, gel permeation with fast protein liquid chromatography (FPLC), and disc polyacrylamide gel electrophoresis. Overall, TRF was purified approximately 34,000-fold with a maximum 3.8% recovery of activity, and the specific activity of the purified TRF was approximately 9.6×10^4 U/mg. The TRF that is active in these systems is distinct from the other lymphokines such as IL 1, IL 2, BCGFI (now known as BSF_{p1}), and γ -interferon. The TRF is extremely hydrophobic, with an apparent m.w. of 50,000 to 60,000 on gel permeation chromatography and 18,000 on SDS-PAGE under reducing conditions. Highly purified B151-TRF abrogated the activity by treatment with trypsin but not with RNase. Moreover, it bound to lima bean agglutinin-Sepharose specific for N-acetylgalactosamine residues, indicating that B151-TRF is a glycosylated glycoprotein containing N-acetylgalactosamine residues. The role of N-acetylgalactosamine residues on TRF activity was additionally substantiated by the fact that the addition of appropriate amounts of N-acetylgalactosamine in the assay systems for TRF preferentially induced a profound suppression for TRF-mediated PFC responses.

It has been generally postulated that B cell proliferation and differentiation into immunoglobulin(Ig)-secreting cells are regulated by several soluble factors derived from macrophages and T cells (1). Furthermore, there is growing evidence to support the idea that different phases of the B cell response to antigenic stimulation are regulated

by functionally and biochemically distinct factors (2). Because complex cellular assay systems have usually been used in many studies, the precise mechanisms regarding the observed result of increased B cell function are not well understood.

T cell replacing factor (TRF)³ has been shown to induce terminal differentiation of late-developing B cells to antibody-producing cells rather than to augment B cell proliferation (3). A number of laboratories are involved in the purification of soluble factors that exert stimulatory effects on B cell differentiation (4–8). Progress has been limited primarily because of the minute quantities of active material produced by T cells. Therefore, there is little information available on the biochemical properties of B cell differentiation factors such as TRF.

Of particular interest to us are the properties of TRF that regulate the terminal differentiation of B cells, which had been activated *in vivo* by immunization with antigen or *in vitro* by anti-IgM antibody. We previously demonstrated that extensively T cell-depleted splenic B cells from mice, as well as murine chronic B cell leukemic cells (BCL₁), could differentiate into antibody-secreting cells in response to soluble products (TRF) found in the cellfree supernatants (CFS) of a T cell hybridoma (B151K12) established by means of fusion of T thymoma cells and *Mycobacterium*-primed T cells (9–11). Furthermore, we also demonstrated that the responsiveness of B cells to B151-TRF was under X chromosome control, as determined by genetic analysis of the TRF low responsiveness of B cells from DBA/2Ha mice (12, 13).

Because B151K12 cells constitutively produce TRF, and because the target (BCL₁) cells are monoclonal in nature, there is little ambiguity concerning the origin of the TRF source and the primary target of TRF. To determine the biochemical characteristics of TRF, we have fractionated samples of B151K12-CFS by using a variety of biochemical techniques. The resulting sets of fractions were then tested for TRF activity that had been previously observed on antigen-activated B cells and BCL₁ cells, and several other lymphokine activities were tested as controls.

In this report, we describe the purification method used to enrich TRF activity from the CFS of a murine T cell

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³Abbreviations used in this paper: CFS, cellfree supernatant; TRF, T cell replacing factor; B151-TRF, TRF in CFS from B151K12 T cell hybridoma; FS6-CFS, CFS from concanavalin A-stimulated FS6-14.13 cells; LBA, lima bean agglutinin; LcH, lentil lectin; WGA, wheat germ agglutinin; TEMED, N,N,N',N'-tetramethylethylenediamine; PFC, plaque forming cell; BCGFI, anti-IgM antibody-dependent B cell growth factor; ATS, anti-thymocyte serum.

hybridoma (B151K12), and we discuss some of the chemical and immunologic properties of the purified TRF.

MATERIALS AND METHODS

Animals. BALB/cCrSlc mice were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan. All animals were used at 5 to 8 wk of age.

Reagents. Keyhole limpet hemocyanin (KLH; Calbiochem Corp., San Diego, CA) and hen egg albumin (OVA; Sigma Chemical Co., St. Louis, MO) were used as carrier proteins. 2,4-Dinitrophenyl (DNP) groups were introduced into those proteins by described methods (8), and DNP₉-KLH and DNP₄-OVA were prepared. Subscripts refer to the average number of DNP groups per molecule of protein. Various lectin-conjugated agarose gels (LBA-gel, Lch-gel, and WGA-gel)³ were obtained from E. Y. Laboratories (San Mateo, CA). Various monosaccharides were obtained from Sigma.

Antisera. Monoclonal anti-Thy-1.2 antiserum (clone F7D5) was obtained from Olac 1976, Bicester Oxon, England. Rabbit antibody specific for mouse μ -chain was raised by immunizing purified IgM secreted by myeloma MOPC104E cells.

BCL₁ cells. BCL₁ tumor, originally donated by Dr. Vitetta (University of Texas Health Science Center at Dallas, TX), was maintained by *in vivo* passage in BALB/c mice. BCL₁ cells were prepared from mice that had carried the BCL₁ tumor more than 4 wk, according to the method described (11). BCL₁ cells were selected by discontinuous Percoll gradient centrifugation.

Preparation of CFS of T cell hybridomas. CFS from a T cell hybrid clone, B151K12, established by means of fusion between *Mycobacterium*-primed T cells and BW5147 thymoma cells, was prepared according to the described method (9). In brief, B151K12 cells were inoculated into Falcon plastic dishes (100 mm diameter, Falcon No. 3001) containing 20 ml of RPMI 1640-10% fetal calf serum (FCS) in an inoculum size of 1 to 3 × 10⁶ cells/dish, were cultured in a CO₂ incubator at 37°C for 24 to 48 hr without stimulation by purified protein derivative (PPD) or by mitogen, and their culture supernatants were collected. CFS from concanavalin A-stimulated FS6-14.13 cells were prepared according to the method described by Harwell *et al.* (14).

Ammonium sulfate precipitation. CFS derived from B151K12 cells were fractionated by ammonium sulfate precipitation at 4°C. Sufficient (NH₄)₂SO₄ powder was slowly added to the supernatants to achieve 50% saturation. The pH of the supernatant was then adjusted to pH 7.0 with NaOH. Stirring was continued for 1 hr, after which the supernatant was centrifuged at 10,000 × G for 20 min. The precipitates were discarded. The precipitation process was then repeated on the resulting supernatant to prepare precipitates in 50 to 85% (NH₄)₂SO₄ saturation. Samples were then either dialyzed against phosphate-buffered saline (PBS) for biologic assay or against 10 mM Tris-HCl buffer (pH 8.5) in preparation for DE52 column chromatography.

DEAE-cellulose column chromatography. Ion exchange column chromatography was conducted in a 22 × 450 mm DEAE-cellulose (DE52, Whatman) column that had been equilibrated in 10 mM Tris-HCl buffer (pH 8.5). The sample that had been prepared by ammonium sulfate precipitation of the B151K12 culture supernatant and dialyzed against the above buffer was applied to the column at a flow rate 20 ml/hr. The sample was washed into the column with 5 column vol of starting buffer. A linear gradient was then initiated by using a final buffer of 0.25 M NaCl in 10 mM Tris-HCl buffer (pH 8.5). An aliquot of each fraction was dialyzed against RPMI 1640 and was assayed for TRF activity.

Blue-Sepharose column chromatography. The concentrated samples with TRF activity from DE52 column chromatography were extensively dialyzed against 50 mM Tris-HCl buffer (pH 8.0) and were applied on a Blue-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) column (10 × 50 mm). Nonbound fractions were collected. After extensive washings, materials adsorbed to the column were eluted with 2 M KCl in 50 mM Tris-HCl buffer (pH 8.0).

Hydroxylapatite column chromatography. Samples containing TRF from Blue-Sepharose column chromatography were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.7) containing 0.2 M NaCl, and were applied onto a hydroxylapatite (Bio Rad Laboratories, Richmond, CA) column (22 × 85 mm) that was equilibrated with the above buffer. A linear gradient was then initiated by using a final buffer of 0.2 M sodium phosphate buffer (pH 6.7) containing 0.2 M NaCl. A sample of each fraction was diluted in PBS containing 5% FCS, and was assayed for TRF activity.

Gel-permeation column chromatography. Samples containing TRF from the hydroxylapatite column chromatography step were pooled and dialyzed against 10 mM HEPES buffer (pH 7.0) in 0.15 M NaCl, were applied onto TSK gel columns (G3000SW; Toyo Soda Co.

Ltd., Atsugi, Japan) that were equilibrated with the above buffer, and were eluted with the same buffer by using fast protein liquid chromatography system (FPLC; Pharmacia).

Tris-glycine polyacrylamide gel electrophoresis (PAGE). Samples containing TRF after sequential ammonium precipitation, DE52 column chromatography, Blue-Sepharose column chromatography, hydroxylapatite column chromatography, and gel permeation on TSK columns were additionally fractionated by Tris-glycine PAGE according to the method described by Davis (15). The sample (5 to 10 μ l) was electrophoresed at 4°C with a running buffer system of pH 8.3 Tris-glycine. The resolving 5 × 90 mm gels contained 7.5% acrylamide, TEMED,³ and 0.07% ammonium persulfate. The stacking gels were 0.3 ml per tube, and consisted of 3.0% acrylamide, TEMED, and 0.005% riboflavin. The gels were run at 2.0 mA per tube, and the electrophoresis was terminated when the tracking dye (bromophenol blue) was 3 mm from the anode end of the gel. The gels were sliced into 2.0 mm slices and were then eluted into 1.0 ml of PBS containing 0.5% bovine serum albumin (BSA) by placing the slice into a dialysis bag and dialyzing against PBS. The eluted material from each fraction was then assayed for TRF, BCGFI,³ IL 2, and interferon (IFN) activity, respectively, according to the methods described below.

SDS-PAGE analysis. SDS-PAGE analysis was conducted according to the method described by Laemmli (16). In brief, samples were dialyzed twice with SDS sample buffer, were supplemented with 5% 2-mercaptoethanol (for reducing gels only) and 10% glycerol, and were electrophoresed with a running buffer system of pH 8.9 on 4 cm 15% polyacrylamide slab gels in 0.1% SDS, until the tracking dye had almost reached the bottom. Prestained m.w. standard proteins (Pharmacia) were also electrophoresed. The gels were sliced into 2.0 mm slices and were then eluted into 1.0 ml PBS containing 0.5% BSA according to the same method as the section above (PAGE).

Enzyme treatment. Partially purified preparations of TRF were treated with either insolubilized trypsin or ribonuclease (RNase). The TRF preparation to be treated was obtained by ammonium sulfate precipitation, DE52 column chromatography, Blue-Sepharose column chromatography, hydroxylapatite chromatography, and gel permeation chromatography. The sample contained over 200 U/ml of TRF and no detectable IL 2 activity. The sample was dialyzed against PBS (pH 7.2) and was treated with either 30 U/ml of insolubilized trypsin or 12.5 U/ml of insolubilized RNase (Sigma) that had been washed in PBS. The reaction mixture was incubated at 37°C for 1 hr on a slowly rotating wheel to prevent the insolubilized enzymes from setting. The factors were harvested by centrifugation, and FCS was added to 1%. The samples were then dialyzed against RPMI 1640 medium overnight, and were assayed for TRF activity as described below.

TRF assay. Two assay systems for TRF activity were employed in the present study: 1) induction of anti-DNP IgG PFC responses in cell cultures of B cells from DNP-KLH-primed BALB/c mice, and 2) induction of IgM PFC in BCL₁ cell culture, as described (8, 13). First, spleen cells from DNP-KLH-primed and boosted mice that had received an injection of anti-thymocyte serum (ATS) 2 days before sacrifice were serially treated with anti-Thy-1.2 antibody plus complement and anti-Lyt-1.2 antiserum plus complement (extensively T cell depleted), and were used as a B cell source. Those DNP-KLH-primed B cells (6 × 10⁵/culture) were co-cultivated with various concentrations of TRF-containing CFS in the presence of DNP-OVA (12 ng/culture). The cultures were set up in triplicate. The numbers of DNP-specific plaque-forming cells (PFC) were enumerated by a modification of the method of Jerne and Nordin (17). Because mainly the IgG class of the anti-DNP PFC response was detected under the conditions employed, only the number of anti-DNP IgG PFC is listed in the *Results*.

Secondly, spleen cells from BCL₁-bearing mice, which had received an ATS injection 2 days before sacrifice, were treated *in vitro* with anti-Thy-1.2 plus complement and anti-Lyt-1.2 plus complement. The recovered cells were layered on discontinuous Percoll gradients (20, 30, and 40%) and were centrifuged at 1800 rpm for 20 min. BCL₁ cells were recovered in the interphase between the 30 and 40% Percoll gradients. The BCL₁ cells (1.5 × 10⁵/culture) were cultured with the appropriate concentrations of CFS of B151K12 in microtiter plates for 3 days. After washing the cultured cells with Hanks' balanced salt solution, the cell suspension was mixed in 0.5% agarose containing SRBC sensitized with protein A. The numbers of IgM-producing cells were enumerated by a reverse PFC assay by using rabbit anti-mouse IgM for the facilitating antibody, as described (8).

An arbitrarily chosen unfractured B151K12-CFS was defined as containing 20 U/ml and was used as a standard in every assay. This standard TRF preparation induced maximal PFC responses up to dilutions of 1/6 to 1/3, as measured by using DNP-primed B cells,

and 1/10 and 1/20, as measured by using BCL₁ cells. In each experiment, a dose-response titration of unfractionated B151K12 supernatant was also performed. A unit of TRF activity in each fraction was defined as the reciprocal dilution yielding a PFC response that was 50% of the maximal response to a standard TRF preparation.

Anti-IgM-dependent B cell growth factor (BCGFI) assay. Samples were assayed for BCGFI (now known as BSF₁) in a co-stimulator assay with anti-IgM, as described (18). Purified B cells were prepared from BALB/c mice by modification of the procedures described by Leibson *et al.* (19) as well as Howard *et al.* (18). The cells were plated at a final concentration of 2.5×10^5 cells/ml (5×10^4 cells/0.2 ml culture) and were stimulated with rabbit IgG anti-mouse μ -chain at a final concentration of 2 μ g/ml, with or without BCGFI, for 3 days. The cells were pulsed with tritiated thymidine (³H-TdR) for the last 18 hr of the incubation period, and were assayed for ³H-TdR incorporation as described (9). A unit of BCGFI is defined as the reciprocal of the dilution yielding a response, in counts per minute (cpm), that is 50% of the maximal response to a standard BCGFI preparation.

IL 2 assay. IL 2 activity was determined by a modified microassay method with the use of IL 2-dependent murine cloned cytotoxic T cells (GY 1 cell line) according to the method described by Stadler *et al.* (20). A unit of IL 2 is defined as the reciprocal of the dilution yielding a cpm response that is 50% of the maximal response to a standard IL 2 preparation.

IFN assay. IFN activity was assayed on mouse L929 cells by the 50% plaque reduction method, as described by Epstein *et al.* (21). The CFS of PPD-stimulated, mouse *Mycobacterium*-primed spleen cells were used as the standard IFN preparation (17 U/ml).

Statistical analysis. Individual experiments were repeated at least four times. The numbers of PFC/culture were logarithmically transformed, and the geometric means and standard errors were calculated. Group comparisons were made by employing Student's *t* test.

RESULTS

The CFS derived from a T cell hybridoma (B151K12) that had been established by means of fusion between *Mycobacterium*-primed T cells with BW5147 thymoma cells was used as a source of TRF, because B151K12 cells constitutively produce TRF without secreting measurable amounts of IL 2 or BCGFI (9, 22). The biochemical characterization of TRF is based on two assay systems: 1) induction of anti-DNP IgG PFC responses in cell cultures of B cells from DNP-KLH-primed mice, and 2) induction of IgM PFC in BCL₁ cell cultures. In each experiment, a dose-response curve titration of unfractionated CFS of B151K12 cells was performed. One of the representative results for the TRF assay is shown in Figure 1. First, spleen B cells from DNP-KLH-primed mice that had been pretreated with ATS 2 days before sacrifice, followed by sequential treatment *in vitro* with anti-Thy-1 antibody plus complement and anti-Lyt-1 antibody plus complement, were tested for the ability to respond to the CFS of B151K12, resulting in anti-DNP IgG PFC. As it is evident from Figure 1A, the addition of CFS of B151K12 alone to the culture of DNP-primed B cells in the presence of DNP-OVA gave rise to a significant number of PFC in a dose-dependent manner after 5 days of culture. Under these conditions, Con A-stimulated, FS6-14.13-derived CFS alone (which contains at least IL 2 and BCGFI) was not sufficient to reconstitute the PFC response.

Secondly, Percoll-purified BCL₁ cells were cultured with CFS from various T cell hybridomas for 3 days, and then the number of IgM PFC was enumerated by a reverse PFC assay. As shown in Figure 1B, the addition of B151K12-CFS induced polyclonal IgM secretion by Percoll-purified neoplastic B cells from the BCL₁ tumor without any anti-Ig stimulation. In contrast, the supernatant from FS6-14.13 did not induce any polyclonal IgM secretion under the same conditions. These results are in

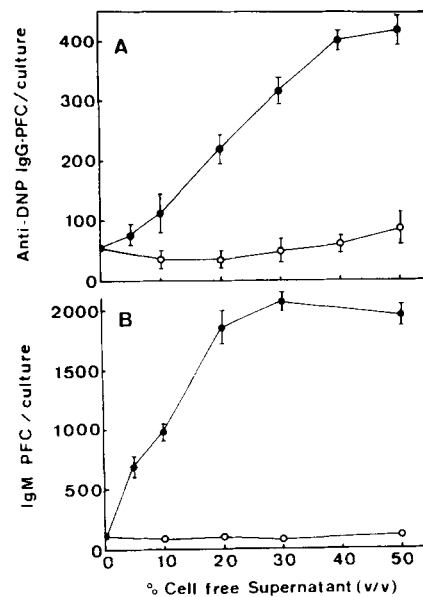


Figure 1. Representative dose-response curves for the TRF activity by using unfractionated CFS of B151K12 cells (●—●) or of FS6-14.13 cells (○—○). A. Anti-DNP IgG PFC responses of DNP-KLH-primed B cells; B. IgM PFC responses of BCL₁ cells.

agreement with our previous observations (9, 11), and also indicate that the addition of unfractionated B151K12 supernatant, but not FS6-14.13 supernatant, could induce differentiation of both DNP-primed B cells and neoplastic B leukemic (BCL₁) cells into antibody-secreting cells in a dose-dependent manner. Moreover, assay systems with the use of BCL₁ cells for TRF activities are more suitable than those with the use of DNP-primed B cells to obtain positive responses in short periods of time (2 days). An arbitrarily chosen unfractionated B151K12-CFS was defined as containing 20 U/ml, because a 1/20 dilution of a test sample gave approximately 50% of the maximal response when using the BCL₁ assay (Figure 1B).

Purification of B151-TRF. The B151K12 cells were cultured in RPMI 1640 medium containing 10% FCS and 5×10^{-5} M 2-mercaptoethanol for 2 days, and 2 to 3 liters of CFS were harvested per week. CFS of B151K12 was salted out with $(\text{NH}_4)_2\text{SO}_4$ in the range of 50 to 85% saturation. Most of the TRF activity was recovered in this fraction. After extensive dialysis against 10 mM Tris-HCl buffer (pH 8.5), samples were applied to a DE52 column and were eluted with a 0 to 0.25 M NaCl linear gradient in 10 mM Tris-HCl buffer (pH 8.5). The TRF activity at each fraction was determined by using DNP-primed B cells or BCL₁ cells. One of the representative results is shown in Figure 2. A single peak of TRF activity was observed that eluted at approximately 75 mM NaCl. Because TRF activity was recovered from fractions before elution of major protein peaks, TRF-active fractions represented a 340-fold increase in specific activity, with a 67% recovery.

Because TRF activities titrated by using DNP-primed B cells were detected at the same fractions as those assayed by using BCL₁ cells, the following assay for TRF activity at various purification steps was conducted by using BCL₁ cells alone, and the results are expressed as units of TRF activity per milliliter, calculated as described in *Materials and Methods*.

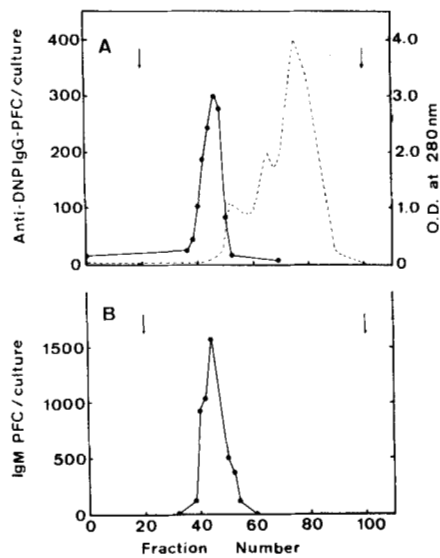


Figure 2. Ion exchange chromatography of TRF activities. Fifty milliliters of dialyzed samples that had been obtained from ammonium sulfate precipitation (50 to 85% saturation) from 3000 ml of B151K12 supernatant were fractionated by DEAE-cellulose chromatography, as described in *Materials and Methods*, and both TRF activity (●—●) and absorbance at 280 nm (----) at each fraction were assessed. TRF activity at each fraction was assayed on DNP-KLH-primed B cells (A) and BCL₁ cells (B), respectively. Arrows indicate the starting and ending points of the elution gradient, which was linear from 0 to 250 mM NaCl in 10 mM Tris-HCl buffer, pH 8.5.

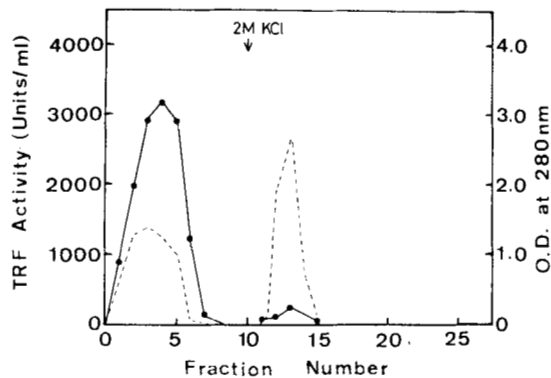


Figure 3. Blue-Sepharose column chromatography of TRF activities. The results are expressed as units of TRF activity per fraction. Ten milliliters of concentrated samples after DE52 chromatography were applied on a Blue-Sepharose column, as described in *Materials and Methods*, and both TRF activity (●—●) and absorbance at 280 nm (----) at each fraction were assessed. Materials bound to Blue-Sepharose were eluted with 2 M KCl in 50 mM Tris-HCl buffer, pH 8.0. The TRF activity of each fraction was titrated by using BCL₁ cells.

The TRF-active fraction from the DEAE column step was concentrated, dialyzed, and applied to a Blue-Sepharose column. Nonbound materials to the Blue-Sepharose column were recovered, and bound materials were eluted with 2 M KCl in 50 mM Tris-HCl buffer (pH 8.0). The TRF activity and protein profile obtained are shown in Figure 3. Most of the TRF activity was recovered from nonadsorbed fractions to Blue-Sepharose.

The TRF-active fractions from the Blue-Sepharose column were dialyzed against 10 mM sodium phosphate buffer (pH 6.7) containing 0.2 M NaCl, and were then applied to a hydroxylapatite column and were eluted with a 10 to 200 mM linear gradient of sodium phosphate buffer at pH 6.7 in 0.2 M NaCl. TRF activity of each fraction was tested by using BCL₁ cells. Most of the TRF activity was recovered in the fractions eluted with approximately 70 mM sodium phosphate buffer (pH 6.7)

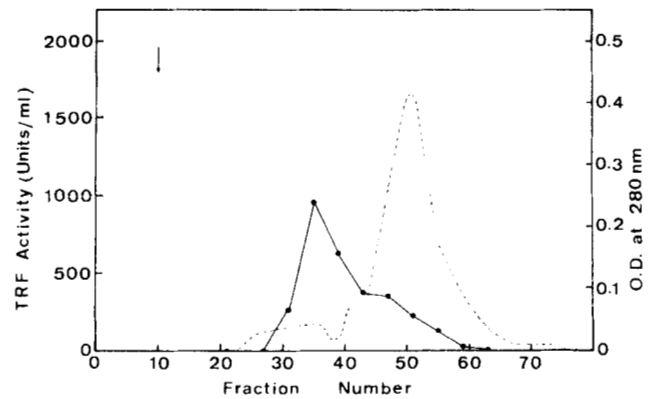


Figure 4. Hydroxylapatite column chromatography. The dialyzed sample of the Blue-Sepharose eluate was additionally fractionated on a hydroxylapatite column, as described in *Materials and Methods*, and both TRF activity (●—●) and absorbance at 280 nm (----) at each fraction were assessed. The TRF activity was determined by BCL₁ cells. Arrows indicate the starting points of the elution gradient, which was linear from 10 to 200 mM sodium phosphate buffer, pH 6.7 in 0.2 M NaCl.

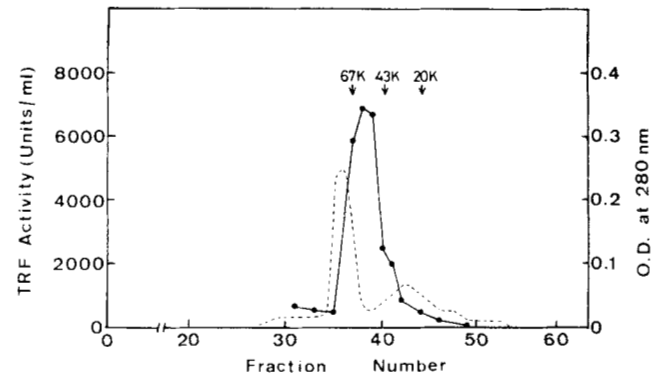


Figure 5. Gel permeation chromatography. The active fraction from the hydroxylapatite column were concentrated and additionally fractionated on a TSK column (G3000 SW), as described in *Materials and Methods*. The positions of apparent m.w. of reference proteins BSA (67,000), OVA (43,000), and soybean trypsin inhibitor (20,000) are shown by arrows. Shown are TRF activity (●—●) and absorbance at 280 nm (----).

(Fig. 4).

To examine the hydrophobicity of partially purified B151-TRF at this stage, the TRF-active fractions from the hydroxylapatite column step were dialyzed against 0.1% trifluoroacetic acid (TFA) and were applied on a ProRPC HR5/10 column (Pharmacia) connected with FPLC and eluted with a 0 to 80% acetonitrile linear gradient in 0.1% TFA. After extensive dialysis against RPMI medium, the TRF activity at each fraction was determined. The TRF activities were recovered at the fraction between 44 to 48% of acetonitrile in 0.1% TFA, suggesting that B151-TRF is extremely hydrophobic in nature (data not shown).

To purify B151-TRF by gel permeation chromatography, TRF-active fractions from the hydroxylapatite column step were concentrated, dialyzed, and applied on a TSK (G3000 SW) column connected with FPLC. The results of TSK-FPLC run in 10 mM HEPES buffer at pH 7.0 in 0.15 M NaCl showed one major and one minor 280 nm absorbing peak. The TRF activity was recovered at the fractions behind the major 280 nm absorbing peak, and it corresponded to an apparent m.w. of 50,000 to 60,000 (at peak 54,000), as shown in Figure 5.

The TRF-active fraction from the gel permeation column step was concentrated and applied to disc polyacryl-

amide gel electrophoresis. After electrophoresis, the gels were sliced at every 2 mm, were eluted in PBS containing 0.5% BSA overnight, and were assayed for TRF activity by using BCL₁ cells. The results are shown in Figure 6. Two bands, one major and one minor, were observed by staining the gels with Coomassie Brilliant Blue. More than 65% of the applied TRF activity was recovered in association with the minor band. In this step, recovery of the protein was arbitrarily calculated by the method based on the ratio of each area corresponding to absorbing patterns at 550 nm. Overall, B151-TRF was purified approximately 34,000-fold, with a maximum 3.8% recovery of activity (Table I). The materials at this point were used for characterization of TRF.

To estimate the apparent m.w. under the reducing conditions, we ran the samples of the five-step-purified B151-TRF on SDS-PAGE. After electrophoresis, portions of the resulting gels were used for silver staining to detect protein, and rest of the gels were sliced every 2 mm, were eluted in PBS containing 0.5% BSA overnight, and were assayed for TRF activity. As shown in Figure 7, the biologic activity of B151-TRF resides at apparent m.w. of 48,000, 36,000, and 18,000 under nonreducing conditions, and it migrates at approximately 18,000 under reducing conditions. At least three bands were visible in the silver-stained gel (under reducing conditions), including one compatible (approximately 18,000 daltons) with

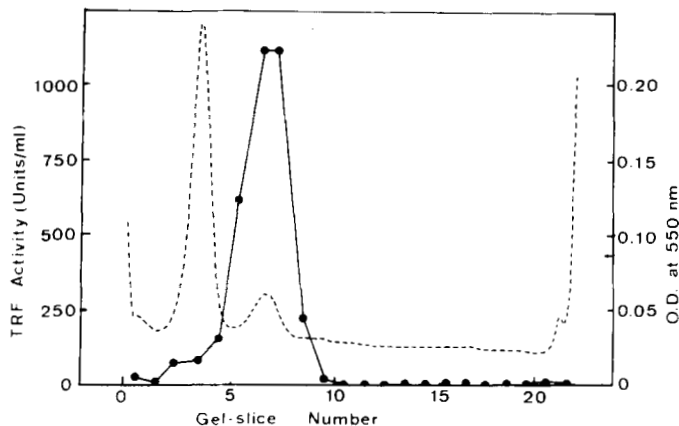


Figure 6. Polyacrylamide gel electrophoresis. The active fractions from the gel permeation chromatography were concentrated and additionally fractionated by polyacrylamide gel electrophoresis, as described in *Materials and Methods*. After electrophoresis, a portion of the gels were stained with Coomassie Brilliant Blue and were monitored by a densitometer at 550 nm (----). The TRF activity (●—●) in eluted materials from each gel slice was determined by BCL₁ cells.

the TRF activities demonstrated in Figure 7 (Fig. 8). By contrast, at least two bands (apparently 48,000 and 36,000 daltons) were visible under nonreducing conditions, each of which is compatible with the TRF activities in Figure 7 (data not shown). Comparison of the staining intensities of these bands with a series of tracks containing known amounts of marker proteins allowed rough calculations of the amounts of protein present. If one assumes that the indicated bands in Figure 8 are indeed B151-TRF, BCL₁ assays would detect TRF activity at concentrations of about 10^{-11} M.

Characterization of B151-TRF. To confirm that purified material from B151K12-CFS exerts only TRF activity, other nominal lymphokine activities of highly purified B151-TRF were analyzed. BCGFI activity was measured by co-stimulating assay by using purified B cells with suboptimal doses of soluble anti-IgM antibody. IL 2 activity was determined by using the IL 2-dependent long-term-cultured CTLL line. IFN activity was determined by a standard blocking effect on virus replication in a cultured murine fibroblast cell line (L929) (20). As can be seen in Table II, BCGFI, IL 2, and IFN activities were not detectable in highly purified B151-TRF preparations, indicating that B151-TRF is a distinct molecule from BCGFI, IL 2, and IFN.

To characterize the molecular nature of B151-TRF, TRF was treated with protease and nuclease, respectively, and the remaining TRF activity was measured. As can be seen in Table III, TRF activity was abolished by the treatment with trypsin, whereas RNase treatment did not affect the TRF activity, indicating that B151-TRF is protein in nature. The stability of B151-TRF to acid treatment or heat treatment was also examined. B151-TRF was stable to dialysis against glycine-HCl buffer at pH 2.0 for 18 hr, and was also stable to heat treatment (56°C, 30 min).

Functional characterization of B151-TRF. Because it is reported that various lymphokines have binding affinity to plant lectin, the TRF-adsorbing activity of insolubilized lectin was examined. The partially purified B151-TRF was applied to LBA-, LcH-, or WGA-coupled-agarose gel columns. Effluent fractions from each gel were collected, and the adsorbed materials were eluted with the relevant monosaccharide from each column. TRF activity in each fraction from each column was tested. One of the representative results is shown in Table IV. Most of the TRF activity was recovered in effluents from both the LcH-gel and the WGA-gel, whereas little TRF activity was

TABLE I
Purification of mouse TRF from B151K12 T cell hybrid cell culture^a

Purification Step	Total Protein ^b (mg)	TRF Activity (1×10^4 , units)	Specific Activity (units/mg)	Purification (fold increase)	Recovery (%)
Starting material	21.000	6.00	2.86	1	100
Ammonium sulfate fraction	10.120	5.67	5.60	1.96	94.5
DEAE-cellulose chromatography	40.5	4.02	9.93×10^2	347	67
Blue-Sepharose column	16.2	3.68	2.27×10^3	794	61
Hydroxylapatite column	1.51	2.09	1.38×10^4	4,839	35
Gel permeation on TSK(G3000 SW)	0.24	1.01	4.21×10^4	14,714	16
Polyacrylamide gel electrophoresis	0.024	0.23	9.58×10^4	33,508	3.8

^a Supernatant was prepared with 10% FCS and was 3000 ml in vol.

^b Protein concentrations were determined relative to BSA standards by using the Bio-Rad protein assay.

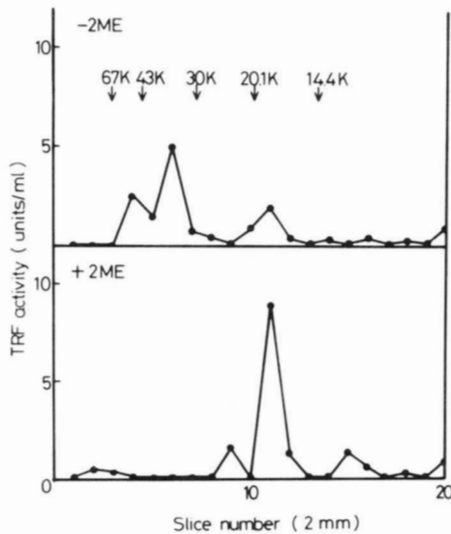


Figure 7. SDS-PAGE analysis of partially purified B151-TRF. The TRF active sample from gel permeation column chromatography was analyzed with SDS-PAGE under nonreducing (upper panel) or reducing conditions (lower panel), according to the methods described in *Materials and Methods*. TRF activity in eluted materials from each gel slice was assayed by using BCL₁ cells.

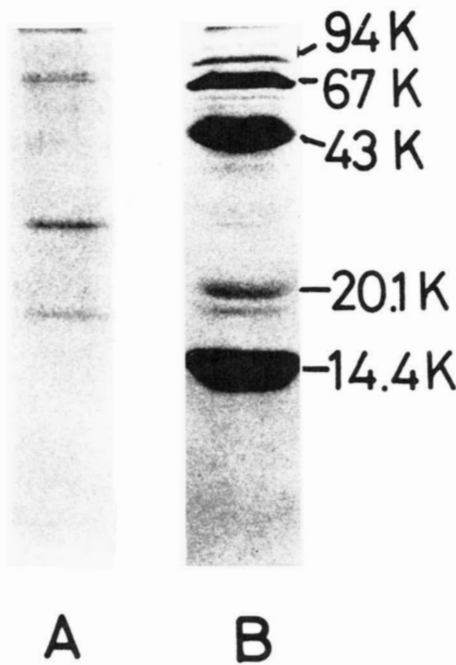


Figure 8. SDS-PAGE followed by silver staining of partially purified B151-TRF. A. The sample of partially purified B151-TRF under the reduced conditions in Figure 7; B. Standard marker proteins.

TABLE II
Lymphokine activities in highly purified B151-TRF

Factors	Lymphokine Activities (units/ml)			
	TRF	BCGFI	IL 2	IFN
B151-TRF	200.0	<0.5	<0.5	<1.0
FS6-CFS	<0.5	18.0	8.0	ND ^b
PPD-CFS ^a	16.0	<0.5	12.0	17.0

^a CFS of purified protein derivative-stimulated *Mycobacterium*-primed spleen cells.
^b Not done.

detected in effluent from the LBA-gel. Moreover, more than 60% of the original TRF activity was recovered from the LBA-gel by eluting with 0.2 M *N*-acetylgalactosamine. By contrast, eluates from the LcH gel with 0.2 M α -

TABLE III
Abolishment of TRF activity by digestion with trypsin but not with RNase^a

	TRF Source	Treatment	TRF Activity	Percent Loss of Activity
Expt. 1.	B151K12	None	200	0
		Trypsin	6	98
		RNase	208	-4
Expt. 2.	B151K12	None	200	0
		Acid dialysis (pH 2.0)	180	10
		Heat treatment (56°C, 30 min)	184	8

^a In experiment 1, the B151-TRF (200 U/ml) was treated with trypsin or RNase according to the method described in *Materials and Methods*. In experiment 2, the same batches of B151-TRF were dialyzed against 50 mM glycine-HCl (pH 2.0) for 18 hr or were treated with heat at 56°C for 30 min. The residual TRF activity was measured by using BCL₁ cells. The results are expressed as geometric means of TRF activity (units/milliliter).

TABLE IV
Binding of TRF to various insolubilized lectin gels^a

Lectin Agarose	TRF Activities (units/ml)		Percent Binding
	Effluent	Eluate	
LBA	26	128	59
LcH	196	18	7
WGA	182	21	16

^a TRF-active fractions (250 U/ml) from gel permeation chromatography were applied to various lectin-conjugated agarose gels (4 ml gel) and the effluent fractions were collected. The materials adsorbed on LBA-gel, LcH-gel, and WGA-gel were eluted with 0.2 M *N*-acetylgalactosamine, 0.2 M α -D-mannose or 0.1 M *N*-acetylglucosamine, respectively. After extensive dialysis of each sample, the TRF activity was measured by using BCL₁ cells, and total TRF activities recovered at each fraction were calculated.

methylmannoside or from the WGA-gel with *N*-acetylglucosamine did not show significant TRF activity. Taking all of the results together, it is most likely that B151-TRF contains *N*-acetylgalactosamine residues in the molecule.

Because B151-TRF contains *N*-acetylgalactosamine residues, it is worthwhile to test whether the *N*-acetylgalactosamine residue plays an important role in the expression of TRF activity. One of the approaches employed here was to investigate the blocking effect of various monosaccharides on B151-TRF-induced PFC responses. As a control, the effect of monosaccharides on the anti-2,4,6-trinitrophenyl (TNP) IgM PFC response induced by TNP-LPS was also examined. One of the representative results is shown in Table V. The addition of 10 mM *N*-acetylgalactosamine to the cultures for TRF assay induced profound suppression of anti-DNP IgG PFC responses mediated by B151-TRF, whereas neither *N*-acetylglucosamine nor fucose inhibited TRF-induced PFC responses. However, the same concentrations of *N*-acetylgalactosamine did not inhibit anti-TNP PFC responses induced by TNP-LPS, suggesting that *N*-acetylgalactosamine residues of B151-TRF may play an important role in the expression of TRF activity.

DISCUSSION

The present experiments defined a molecule (TRF) that induces differentiation and/or maturation of B cells from DNP-KLH-primed mice, as well as chronic B leukemic cells, to antibody-secreting cells. Because the B151K12 cells, when grown in a RPMI 1640 medium containing 2 to 10% FCS and 5×10^{-5} M 2-mercaptoethanol, produce constitutively TRF (10), we chose CFS of B151K12 cells

TABLE V

Blocking effect of various monosaccharides on *in vitro* PFC responses

Stimulants	Monosaccharide ^a	Anti-DNP IgG PFC per Culture	Anti-TNP IgM PFC per Culture
None	—	68 (1.02)	
B151 ^b	None	548 (1.09)	ND ^c
	<i>N</i> -acetylgalactosamine	79 (1.11)	
	<i>N</i> -acetylglucosamine	596 (1.06)	
	Fucose	502 (1.11)	
None	—		28 (1.14)
TNP-LPS ^d	None		363 (1.04)
	<i>N</i> -acetylgalactosamine	ND	274 (1.02)
	<i>N</i> -acetylglucosamine		382 (1.05)
	Fucose		302 (1.09)

^a Concentrations of monosaccharides were *N*-acetylgalactosamine, 10 mM; *N*-acetylglucosamine, 10 mM; and fucose, 10 mM.

^b DNP-primed B cells were cultured with highly purified B151-TRF (20 U/ml) (after gel permeation step) in the presence of or in the absence of various monosaccharides. All of the cultures were stimulated with DNP-OVA (12 ng/culture) and were assayed for anti-DNP IgG PFC at day 5.

^c Not done.

^d Normal B cells were stimulated with TNP-LPS (1 µg/ml) in the presence or absence of various monosaccharides and were assayed for anti-TNP IgM PFC at day 4.

as a starting material for TRF purification. The TRF has been sequentially purified from CFS by DEAE-cellulose chromatography, a Blue-Sepharose column, hydroxylapatite chromatography, gel permeation column chromatography on a TSK column (G3000 SW) with a FPLC system, and disc polyacrylamide gel electrophoresis. Starting from 3 liters of B151K12-CFS, the B151-TRF was purified approximately 34,000-fold, with a final recovery of approximately 3.8% (Table I). This purified material exerted only TRF activity, without any other known lymphokine activities such as BCGFI, IL 2, and IFN (Table II). Because the TRF activity was abrogated by treatment with trypsin but not RNase (Table III), B151-TRF is protein in nature.

The purified B151-TRF isolated in the present study has an apparent m.w. of 50,000 to 60,000, as determined by gel permeation chromatography (Fig. 5) and by Ultrogel AcA 54 column chromatography, as described previously (9), in salt solution. The TRF-active molecules at this purification step are mildly acidic (pI 4.9 to 5.1) (9) and extremely hydrophobic according to our recent analysis by using reverse phase column chromatography with high pressure liquid chromatography.

As shown in Figure 7, the biologic activity of purified B151-TRF after the gel permeation column step resides at an apparent m.w. of both species of 48,000 (a minor peak) and 36,000 (a major peak) in SDS-PAGE analysis under nonreducing conditions, and it migrates as an approximate 18,000 dalton molecule under reducing conditions. These results may imply that native B151-TRF has an apparent m.w. of 50,000 to 60,000 (peak at 54,000) on gel filtration, and dissociates to an 18,000 m.w. species by SDS under reducing conditions. Therefore, we believe the molecule of 18,000 daltons to be a monomer.

Concerning the biologic properties of TRF, the observations that B151-TRF could bind to the LBA-conjugated agarose gel and that the activity was recovered in the fraction eluted with 0.2 M *N*-acetylgalactosamine (Table IV) should be stressed. These results indicate that B151-TRF contains *N*-acetylgalactosamine residues. Furthermore, the addition of *N*-acetylgalactosamine into the as-

say system for TRF by using DNP-primed B cells induced a profound suppression of TRF-mediated anti-DNP IgG PFC responses (Table V), indicating that *N*-acetylgalactosamine residues in the B151-TRF molecule may play an important role for the expression of TRF activity and/or binding of TRF to the acceptor site(s) on target cells.

It is clear that we are far from a complete understanding of the molecular mechanism of T cell-derived factors that govern B cell proliferation and differentiation into antibody-forming cells. Many soluble factors have been reported to affect the later stages of B cell differentiation, as reviewed by Howard and Paul (2). Isakson *et al.* (5) described the existence of two kinds of B cell differentiation factors, termed BCDF μ and BCDF γ . These lymphokines appear to act directly on activated normal B cells (or in the case of BCDF μ , on BCL₁ cells) to induce the synthesis and secretion of IgM or IgG, respectively. According to their results, B151-TRF could only induce secretion of IgM from normal B cells, as well as BCL₁ cells. Therefore, B151-TRF belongs to BCDF μ but not to BCDF γ (5). However, as we reported in preceding papers and also in the present study, B151-TRF induces remarkable IgG PFC responses. The reasons for the discordant results between ourselves and the other investigators may be due to different assay systems, and additional analysis of mechanisms for class-switching from IgM to IgG by using highly purified B151-TRF may contribute to understanding the functional differences between B151-TRF and BCDF γ .

Leibson, *et al.* (19, 23) reported that IFN γ could induce terminal differentiation of B cells in combination with IL 2. Our results distinguish B151-TRF from a number of previously described lymphokines (IL 1, IL 2, BCGFI, and IFN), because the highly purified B151-TRF material did not show any significant BCGFI, IL 2, or IFN activity (Table II). Furthermore, the addition of CFS containing IL 2 and BCGFI to the assay systems for TRF employed in the present study did not induce PFC responses (Fig. 1). Moreover, the addition of IFN γ produced by recombinant DNA technology does not trigger BCL₁ cells for IgM-secreting cells (unpublished observation). These results strongly indicate that the TRF distinct from IL 2 and IFN can induce terminal differentiation of B cells into antibody-forming cells. However, this does not exclude the possibility that IFN γ exerts its activity to B cells in combination with IL 2, giving rise to antibody-secreting cells. It is possible that the B cell population responsive to B151-TRF is distinct from that responsive to IFN γ , because B cells that were activated by anti-IgM antibody and BCGFI responded to B151-TRF and EL-TRF with synergism (22).

Melchers and his associates (4) described antigen-nonspecific helper factors from helper T cell lines inducing the replication and maturation of B cells. They provisionally named those factors as B cell replication and maturation factors (BRMF). According to their results, BRMF act on B blastoid cells that have been activated with helper T cells and antigen in a major histocompatibility complex-restricted fashion. It is too early to compare our B151-TRF with their factors on a molecular basis.

Recently, Sidman and his collaborators (7) reported the initial biochemical characterization and purification of B cell maturation factors (BMF), which is a lymphokine or family of lymphokines promoting the maturation of some

tumor lines of B cell lineage, as well as normal B cells. According to their results, BMF molecules are distinct lymphokine from IL 1, IL 2, granulocyte-macrophage-CSF, IFN and BCGFI, and are probably heterogeneously glycosylated glycoproteins with an apparent m.w. of 50,000 to 55,000 by gel permeation chromatography and 16,000 by SDS-PAGE. As judged from the apparent m.w. of B151-TRF, BMF has very similar features to B151-TRF.

Swain, Dutton, *et al.* (24, 25) recently found B cell growth factor (BCGFII) in CFS from antigen-activated T cell clones or T cell hybridomas. According to their findings, BCGFII activity can be assayed in a co-stimulator assay with normal B cells and dextran sulfate or with the BCL₁ *in vivo* line. Moreover, CFS of B151K12 exerted potent BCGFII activities on BCL₁ cells. Therefore, the question arose in our mind as to whether partially purified B151-TRF exerts BCGFII activity on the BCL₁ *in vivo* line. During the course of the purification procedure, BCGFII activity was also tested. BCGFII activities always resided in the same fraction in which TRF activity was detected. The highly-purified B151-TRF preparation (apparently 18,000 daltons) after SDS-PAGE (under reducing conditions) also showed BCGFII activity (unpublished observation). Therefore, it appears that the B151-TRF analyzed in the present study may exert growth and differentiation signals on B cells. To clarify this point further, the stimulatory effect of purified B151-TRF on the growth and differentiation of B blastoid cells, as well as resting B cells, is under investigation.

Besides the possible existence of various T cell factors responsible for B cell differentiation, we have to locate the functional site of various T cell factors at the sequential steps of B cell differentiation. Only this may enable us to define the real functions of the T cell factors. When a cloned B cell line is available in which the differentiation stage is functionally defined, we will be in a position to determine what kinds of helper T cell factors are required in the process of B cell differentiation. As judged from our results described here, we speculate that B151-TRF acts on at least mature B cells at their terminal differentiation stage to antibody-forming cells.

The studies reported herein provide first step purification of sufficient quantities of TRF to distinguish the active molecule responsible for B cell differentiation. At present, studies are in progress to additionally characterize the molecule with respect to its primary structure, as well as its role for B cell differentiation and/or maturation.

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