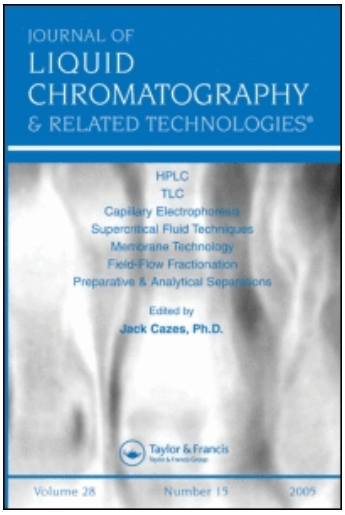

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The Use of High Performance Molecular Sieving Columns for the Study of Lymphocyte Products. I. Macrophage Transglutaminase Interaction with Products of Con A-Stimulated Mouse Spleen Cells

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THE USE OF HIGH PERFORMANCE MOLECULAR SIEVING COLUMNS FOR THE STUDY OF LYMPHOCYTE PRODUCTS

I. MACROPHAGE TRANSGLUTAMINASE INTERACTION WITH PRODUCTS OF CON A-STIMULATED MOUSE SPLEEN CELLS

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ABSTRACT

Partially purified transglutaminase from mouse peritoneal macrophages has been prepared and shown to utilize mouse

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lymphokines as substrates. Biosynthetically labeled mitogen-stimulated spleen cell products were fractionated by high performance liquid chromatography on a molecular sieving column. A fraction known to contain macrophage migration inhibition factor (MIF) of molecular weight 15,000 kd was reacted with the transglutaminase and rechromatographed. Higher molecular weight labeled components which did not dissociate in the presence of 6M guanidine HCl were observed. These data suggest that the molecular heterogeneity often reported for lymphokines may be the result of transglutaminase modification of their native structures. The relationship of these findings to possible regulatory functions in the immune response is suggested.

INTRODUCTION

Recent data obtained during purification studies on mouse (1) and human (2) macrophage migration inhibition factor (MIF) shows that these activities exists in multiple molecular weight fractions and indicates that polymerization of some type is involved. Preliminary studies using dissociating solvents such as guanidine HCl suggest that this association involves formation of covalent bonds. Since both Concanavalin A (Con A) -stimulated lymphocytes (3) and various macrophages (4) are known to produce a transglutaminase that can form epsilon-(gamma-glutamyl)lysine linkages between susceptible substrates, we felt the presence of

these cell types in biological systems employed normally for generation of MIF might explain this observation. Using HPLC molecular sieving columns and dissociating solvents, we prepared enzyme from murine macrophages and studied its ability to polymerize biosynthetically labeled, mitogen-stimulated spleen cell products, tentatively identified as lymphokines.

MATERIALS AND METHODS

Assay of Transglutaminase Activity. Transglutaminase activity was assayed as described by Schroff, et al (4), a measurement of ^{14}C-putrescine incorporation into casein. The reaction mixture (85 ul) contained final concentrations of 100 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, 10 mM $CaCl_2$, 3 mM dithiothreitol, 4 mg/ml alpha-casein, and 0.65 mM ^{14}C-putrescine (5-10 uCi/mMole). The reaction was initiated by addition of 15 ul of the enzyme fraction and the mixtures were incubated for 2 hr. Control samples were prepared by first heating enzyme preparations to 100C for 10 min. Duplicate aliquots were removed from each vial and placed onto glass fiber filter paper strips that had been pretreated with 0.1% unlabeled putrescine. The filters were dipped immediately into 10% trichloroacetic acid (TCA) and were washed three times by floatation on 5% TCA solutions. The filters were washed in ethanol/acetone 1:1 (v:v) and then in

acetone only and dried. Radioactivity was counted in an LKB liquid scintillation counter. Control values were subtracted and specific activity calculated based on umoles putrescine incorporated/mg/min.

Preparation of Transglutaminase Fractions. Transglutaminase was prepared from thioglycollate induced mouse peritoneal exudates. Peritoneal exudate cells (6.0×10^8) were harvested from 20 mice and allowed to adhere to teflon bags for 2 days in Dulbecco's complete medium supplemented with 5% heat inactivated fetal calf serum. The adherent cells were washed twice in Dulbecco's complete medium without serum. The remaining cells (1.6×10^8) were recovered in 2 ml of lysis buffer consisting of 100 mM Tris-HCl, 50 mM NaCl, 1mM EDTA, and 3 mM dithiothreitol. The cell suspension was lysed by sonic disruption at 4C and clarified by centrifugation at 37,000 x g for 30 min. The transglutaminase-containing supernatant was partially purified by high performance liquid chromatography (HPLC) on a molecular sieving column (Toyosoda TSK 2000SW). The column was equilibrated in 0.05M phosphate buffered saline, pH 7.2, and had been calibrated previously with standard proteins of known molecular weights. The supernatant (200 ul containing 2.8 mg protein) was loaded onto the column and chromatographed at 0.5 ml/min. Fractions were collected every 30 seconds and assayed for

transglutaminase activity as described above. The fractions containing transglutaminase activity were pooled.

Preparation of Biosynthetically Labeled Lymphokines. Biosynthetically labeled lymphokines were prepared from mouse spleen cells as described previously (5). Spleens were perfused with complete Dulbecco's Minimal Essential Medium containing 5% heat inactivated fetal calf serum. After homogenization and filtration through cheesecloth, cells were harvested by centrifugation at 400 x g for 10 minutes and erythrocytes were lysed by osmotic shock. The cell pellet was resuspended in 10 ml of Dulbecco's without FCS and viability and cell count were determined by trypan blue exclusion. Cells were added to 24 well plates at 1×10^7 cells/well in 1.0 ml aliquots. Cells were stimulated by the addition of 10 ugrams of Con A/well and allowed to incubate for 2 hr at 37C in 7% CO_2. Duplicate cultures were prepared without Con A stimulation. After 2 hr, the medium and nonadherent cells were removed and leucine-free medium was added to each well. ^{3}H-leucine (50 uCi) was added to each well and the cells were allowed to incubate for 24 hr. After incubation, the supernatants were harvested and desalted over Sephadex G-25 and lyophilized. The labeled molecules were partially purified by HPLC as described above on a molecular sieving column. Fractions were counted in a liquid scintillation counter and that portion

of biosynthetically labeled material eluting at a molecular weight of approximately 15 kd was used in the remaining studies. Our previous studies have shown the labeled material to co-purify with MIF activity.

Reaction of Lymphokines with Transglutaminase. The 15 kd tritium labeled MIF-containing fraction obtained from HPLC was incubated with the transglutaminase active fraction (approx 80 kd) for 2 hours at 37C and was then rechromatographed on the same HPLC column to determine if the labeled MIF would appear at higher molecular weight ranges because of transglutaminase activity. In order to insure that any changes were due to enzyme activity, controls were prepared in which an excess of putrescine, a substrate for transglutaminase, was added and chromatography was carried out similarly. The covalent nature of the interaction was differentiated from non-covalent association by repeating the chromatographic separation in the presence of 6M guanidine HCl.

RESULTS

Partial Purification of Transglutaminase. The method for partial purification of transglutaminase from thioglycollate induced macrophages was sufficient for these applications. The technique described allowed preparation of sufficient amounts of enzyme with an increase of 38-fold in specific activity. More

importantly, the technique allowed quick separation of transglutaminase activity from other contaminating proteins which enabled us to determine shifts in lymphokine elution in other molecular weight ranges. Figure 1 shows the elution profile of the macrophage cell lysate when chromatographed on the TSK2000SW HPLC column. The major portion of transglutaminase activity elutes at a molecular weight of about 80 kd which is consistent with the reported molecular weight for many types of these enzymes (6).The fraction indicated was used for the remaining studies.

Preparation of Low Molecular Weight Lymphokines. We have previously reported the utility of ^{3}H-amino acid incorporation into mitogen stimulated lymphocyte products and their subsequent identification as lymphokines (5). This technique was used to prepare labeled proteins and a fraction rich in MIF was selected for further study. Figure 2 shows the elution profile of labeled lymphokine on the same molecular sieving column as above. The low molecular weight fraction was collected and incubated with transglutaminase (Fig. 3). After incubation with the enzyme, the lymphokine fraction was observed to elute at higher molecular weights corresponding to those where biological activity for MIF has been reported previously (1). This increase in apparent molecular weight did not occur in an excess of an alternate transglutaminase substrate. To determine whether this change in elution was due to covalent linkage of the small labeled species,

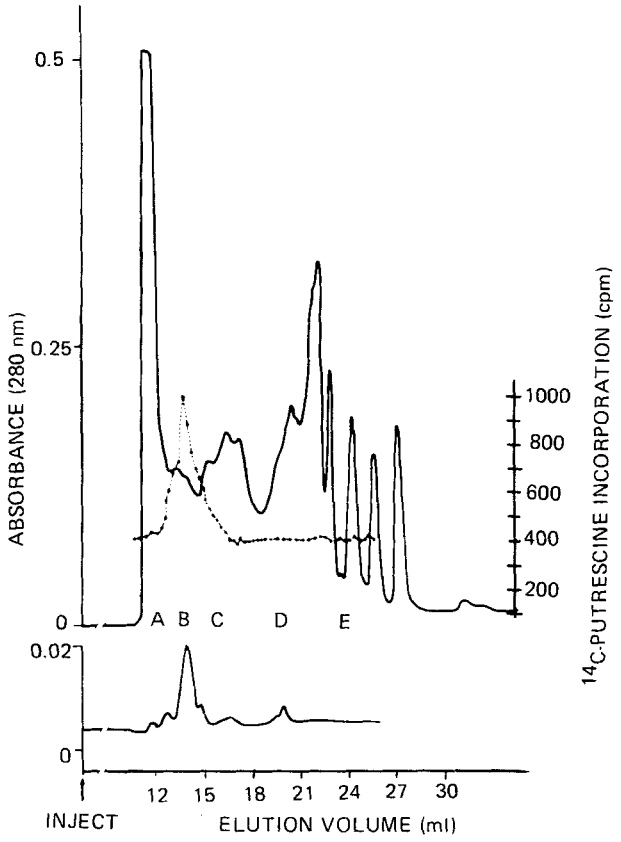

Figure 1. Molecular sieving HPLC of macrophage lysate. Cell lysate (200 ul containing 2.8 mg protein) was loaded onto a 0.75 x 60 cm Toyosoda TSK2000SW column which was pumped with PBS, pH 7.2 at a flow rate of 0.5 ml/min. Optical density was monitored at 280 nm. Fractions (0.25 ml) were collected and assayed for transglutaminase activity as described in the text. The solid line is 280 nm absorbance, the broken line is enzyme activity expressed as ^{14}C cpm. The calibration proteins marked are: A- Thyroglobulin, 670 kd; B- IgG, 158 kd; C- Ovalbumin, 44 kd; D- Myoglobin, 17 kd; and E- Vitamin B-12, 1.35 kd. Transglutaminase activity is shown to elute at approximately 80 kd. The lower portion of the figure shows a sample of the fraction selected for further studies rechromatographed as above.

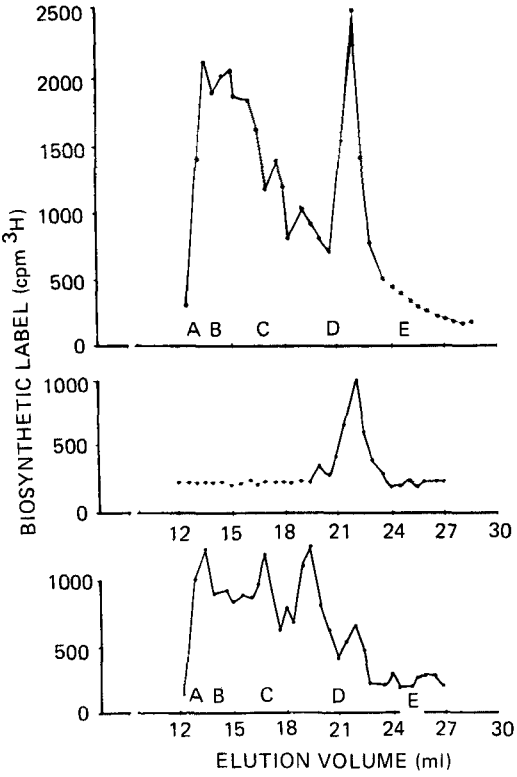

Figure 2. Molecular sieving of biosynthetically labeled lymphokine fractions from mouse spleen cells. ^{3}H-labeled lymphokines were prepared as described in the text and HPLC was performed as in Fig 1. Fractions were collected and counted by liquid scintillation. Fig 2 shows a profile of labeled mitogen treated mouse spleen cell lymphokine supernatants (upper). The fraction at approximately 15 kd was collected and rechromatographed (middle). After incubation of this 15 kd lymphokine with the transglutaminase fraction from Fig 1, the HPLC was repeated (lower). Note the shift toward higher molecular weight as determined by elution position.

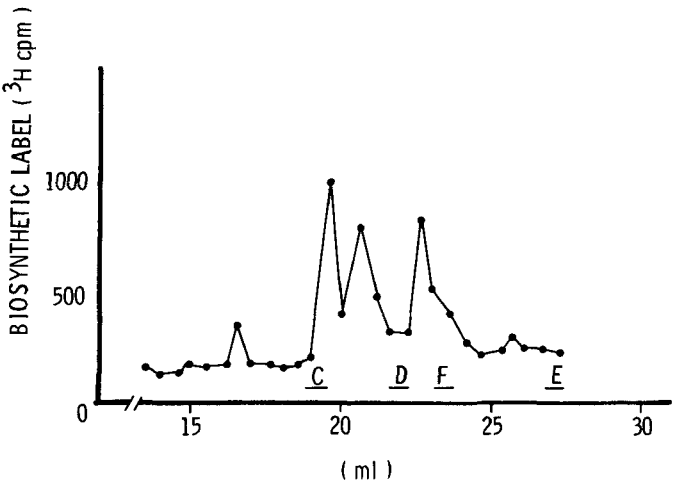

Figure 3. HPLC in the presence of 6M guanidine HCl on a TSK4000SW column. The column was a 0.75 x 60 cm Toyosoda TSK4000SW pumped at 0.5 ml/min with 6 M guanidine HCl. Calibration standards C, D, and E as in Fig 1. F=cytochrome C, 12.5 Kd. Fractions were collected and counted as above. Peaks are at multiples of 15 Kd. Guanidine HCl did not dissociate these higher molecular weight species, suggesting covalent bonds have been formed.

the chromatography was repeated in the presence of 6M guanidine HCl. The higher molecular weight species remained intact, suggesting that their increased size was, indeed, the result of covalent linkage by transglutaminase.

DISCUSSION

The molecular heterogeneity of lymphokines from various systems has been reported often (1,2,9-12). The relationship of these

activities has remained undefined largely because of the lack of sufficient material for exacting chemical analysis. In some instances, higher molecular weight active species are merely complexed with contaminating serum proteins such as albumin and can be readily dissociated by altering solvent composition (8). In the case of murine MIF, these higher molecular weight forms are not dissociated easily and appear to involve covalent bond formation. Sorg has shown that different species of MIF predominate depending upon culture conditions, e.g., as incubation time increases, molecular weight increases (1) as determined by bioassay of Sephadex fractions. Schroff et al have demonstrated that macrophages contain transglutaminase activity and have shown by staining techniques that the enzyme can be used as a marker for macrophage activation (4). The heterogeneity of mouse MIF may be due to its ability to serve as a substrate for the transglutaminase which is present from contaminating macrophages (12).

The present data confirm that covalent bond formation via transglutaminase does increase the molecular weight of biosynthetically labeled proteins present in the supernatant of mitogen-stimulated spleen cells. Because of limitations in bioassay sensitivity, we have not shown that these larger forms are active, yet they do correspond with the elution of bioactive molecules obtained from preparative procedures. It is difficult to determine whether these higher molecular weight forms of MIF

are artifacts of *in vitro* processing, or if they are representative of *in vivo* states that may be regulated by a system in which transglutaminase participates. One could speculate concerning the potential regulatory interaction of lymphokines, polyamines, transglutaminase, alpha-2-macroglobulin, lymphocytes and macrophages. Since all of these elements affect various immune functions (13-18), the ability of both alpha-2-macroglobulin and transglutaminases to bind amines might well be of some regulatory significance.

The present bioassay techniques for MIF and the lack of completely chemically characterized MIF do not allow quantitative comparison of the heterogeneous species; therefore, any further speculation about the physiological significance of these findings is unwarranted. Additionally, appearance of multiple active fractions, although resulting from transglutaminase action, may be an *in vitro* artifact with no *in vivo* basis. The importance of these findings in terms of *in vivo* correlation can be evaluated only when measurement of MIF by direct chemical or immunoassay procedures becomes available.

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