CONNECTIVE TISSUE ACTIVATION

XV. Stimulation of Glycosaminoglycan and DNA Synthesis by a Polymorphonuclear Leukocyte Factor

STEPHEN L. MYERS and C. WILLIAM CASTOR

Human synovial fibroblasts in culture have been stimulated to augment hyaluronate synthesis and glucose utilization by connective tissue activating peptides (CTAP) extracted from human spleen, lymphocytes, platelets, granulocytes, and tumor cells. The platelet-derived mediator CTAP-III also stimulated DNA synthesis in synovial fibroblasts, but CTAP-I from lymphocytes and spleen did not. The present study demonstrates the mitogenic potential of a granulocyte mediator (CTAP-PMN). Normal granulocytes were prepared with Ficoll-diatrizoate gradients, platelet contamination being estimated by phase microscopy and by radioimmunoassay for the platelet-specific protein, β-thromboglobulin. CTAP-PMN preparations derived from 4×10^7 cells/ml stimulated culture ^{3}H-thymidine incorporation to 3.56 ± 1.32 (SD) times control levels. Although exposure of preparations to thiols reduced their mitogenicity, CTAP-PMN was relatively heat-stable. SDS gel electrophoresis of active fractions suggested a molecular weight between 12,700 and 15,700 daltons. In double immunodiffusion, antisera to CTAP-III showed no reactivity with CTAP-PMN. CTAP-PMN or other granulocyte factors capable of stimulating fibroblast DNA synthesis may play a role in chronic proliferative synovitis or in other settings where exudative inflammation is accompanied by connective tissue growth.

Hyaluronic acid is the principle glycosaminoglycan (GAG) produced by synovial fibroblasts, both those lining the joint space and those cultivated in vitro. Several agents which stimulate or "activate" the synthesis of hyaluronate (HA) by cultured synovial fibroblasts have been identified and partially characterized chemically. These agents, denoted "*C*onnective *T*issue *A*ctivating *P*eptides" (CTAP), have been isolated from human lymphocytes (CTAP-I), $HE_{p\text{-}2}$ epithelial tumor cells (CTAP-II), and human platelets (CTAP-III) (1–3). Protease digestion destroys the biologic potency of these mediators, which range in molecular weight from 9,300 to 14,600 daltons. The "activation" response to the CTAP mediators includes stimulation of culture glucose utilization and lactate production, GAG synthesis, and in some cases, stimulation of DNA synthesis. The mechanism by which these agents stimulate HA production involves increased fibroblast RNA and protein synthesis, and increases in prostaglandin E formation and intracellular cyclic AMP levels. CTAP-III is presently the only one of these mediators known to stimulate DNA synthesis in fibroblast cultures. In a rat fibroblast line, similar increases in HA production have been produced by exogenous dibutyryl cAMP and cAMP and by a calf serum factor with a molecular weight of approximately 100,000 daltons (4,5). Plasma somatomedins A and C, serum NSILA-S and P, and several platelet growth factors with molecular weights ranging between 4,700 and 24,000 daltons have been reported to stimulate either GAG synthesis or both GAG synthesis and cell division in cultured connective tissue cells (6).

From the Department of Internal Medicine and the Rackham Arthritis Research Unit, The University of Michigan Medical School, Ann Arbor, Michigan 48109.

Supported by USPHS-Training Grant AM-07080, USPHS-Grant AM-10728, and in part by Institutional Research Grant No. IN-40R to The University of Michigan from the American Cancer Society.

Address reprint requests to C. William Castor, MD, Rackham Arthritis Research Unit, The University of Michigan Medical School, Ann Arbor, Michigan 48109.

Submitted for publication July 20, 1979; accepted in revised form December 20, 1979.

The present study focuses on polymorphonuclear leukocytes, cells which predominate in effusions bathing the synovium in inflammatory arthropathies such as rheumatoid arthritis. In earlier studies, saline extracts prepared from granulocytes purified by adherence to glass beads stimulated HA synthesis, lactate production, and glucose utilization in synovial fibroblast cultures (7). Significant numbers of glass-adherent platelets and monocytes may have contributed to the stimulation observed in these experiments. The current study extends these observations on the role of the granulocyte in connective tissue activation.

MATERIALS AND METHODS

Granulocyte separation. Granulocytes were prepared on a discontinuous Ficoll-diatrizoate gradient similar to that discribed by Böyum (8). Healthy donors provided 60 ml samples of venous blood which was anticoagulated with 0.27% ethylenediaminetetraacetic acid (EDTA). The samples were immediately diluted with 2.5 volumes of a calcium and magnesium-free buffered physiologic salt solution (Rabinowitz's solution) (9). Solutions of 33.9% sodium diatrizoate (Winthrop, New York) and of 9% Ficoll 400 (Pharmacia, Uppsala, Sweden) were mixed in a 1:2.3 ratio by volume to a specific gravity of 1.083 ± 0.001 at 16°C. Thirty-five milliliters of this gradient solution were placed in a 60 × 145 mm centrifuge bottle and overlayered with 100–120 ml of dilute blood. After centrifugation (400*g*, room temperature, 30 minutes), the platelet-mononuclear cell layer was carefully removed and again centrifuged (17,300*g*, 4°C, 10 minutes) to obtain a cell-free supernate. The erythrocytes and granulocytes were removed from the separation bottle and resuspended in this supernatant solution. Dextran (Sigma, St. Louis) was added to a final concentration of 0.8%, and the erythrocytes were allowed to sediment at 4°C. Granulocytes were harvested from the resulting supernatant fluid by low-speed centrifugation (150*g*, room temperature, 10 minutes) and suspended in 0.87% NH_4Cl to lyse remaining erythrocytes. The cells were then washed, counted, and frozen at −70°C. Granulocyte yield ranged between 55% and 85% of the whole blood population, or about 5×10^7 granulocytes/60 ml blood. Differential counts distinguishing mononuclear cells and granulocytes were performed on cell suspensions stained with crystal violet. Enumeration of platelets and leukocytes was carried out with hemocytometers using phase contrast illumination.

Fibroblast culture. Normal synovia obtained at arthrotomy or amputation were divided into explants from which synovial fibroblast strains were cultivated in monolayer, as previously reported (10). Routine culture medium consisted of 80% medium 1066, 10% fetal calf serum, and 10% heat-inactivated pooled human serum with supplementary L-glutamine, penicillin G, streptomycin, and 0.02*M* Hepes buffer, pH 7.4.

Assay for fibroblast "activation." The standard bioassay for fibroblast "activation" in the presence of the CTAP mediators has been previously described (11). Granulocyte extracts and other samples in these assays were dialyzed overnight at 4°C against 0.05*M* phosphate-buffered saline (PBS), pH 7.0, and precipitates which formed during dialysis were removed by centrifugation. Samples were sterilized by Millipore filtration and stored at 0°C. Sufficient T-15 culture flasks were each planted with 10^6 synovial fibroblasts to allow triplicate control and duplicate experimental assay flasks. After cell attachment was complete (4–6 hours), a serum-free assay medium (2 ml Eagle's basal medium with 0.02*M* Hepes buffer, pH 7.4, L-glutamine, penicillin G, and streptomycin) was introduced. Control flasks were charged with 0.3 ml PBS, and samples were added in a similar volume. After 40 hours of incubation at 35–37°C, the medium was removed. Hyaluronic acid in the medium from each flask was isolated by cetylpyridinium chloride precipitation (11) and quantitated by determining uronic acid with a modified carbazole method (12). Cell sheet protein was measured by the method of Oyama and Eagle (13).

Measurement of mitogenic activity. The rate of DNA synthesis in cultured fibroblasts was evaluated in cells incubated with ^{3}H-methylthymidine as described recently (2,3). Aliquots of 10^4 cells were placed in 6.4 mm microtiter wells in an assay medium containing 97% Eagle's standard medium (ESM), 3% fetal calf serum, 0.02*M* Hepes, and L-glutamine. After a 24-hour incubation at 37°C, fresh medium (200 μl) and samples (5–7.5 μCi, 15μl) were added. ^{3}H-methylthymidine (1.5 μl) was added and incubation at 37°C was resumed for an additional 24 hours. The medium was then discarded, and the cell sheets were washed twice with PBS at pH 7.0. After single washes with 5% trichloroacetic acid and absolute methanol, the cell sheets were dried and extracted with 0.3*N* NaOH (50 μl). Extracts were spotted on glass fiber filter paper (Whatman grade 934 AH, H. Reeve Angel Inc., Clifton, New Jersey) and counted in a Beckman Series 7,000 liquid scintillation counter.

Miscellaneous methods. SDS polyacrylamide gel electrophoresis was performed in 15% slab gels. Molecular weight standards as well as other enzymes were obtained commercially (Sigma, St. Louis). Neutral protease activity was detected by measuring color released from a substrate of blue azure dye bound to hide powder during an 18-hour incubation at pH 7.0, 37°C (14). Trypsin (2 × crystallized) was used as a standard. CTAP-III preparations were prepared as previously described (2,3).

Beta-thromboglobulin assay. Radioimmunoassay for beta-thromboglobulin (β-TG) (Amersham, Chicago) was performed with 50% of the reagent volumes per assay tube recommended by the supplier. This did not affect the sensitivity or reproducibility of the standard curve in the range between 10 and 20 ng β-TG/ml. Theophylline (0.18 mg/ml) and 3.3% EDTA were added to blood samples from which normal platelets were collected for β-TG assay to minimize platelet release phenomena, and all samples were measured in duplicate.

RESULTS

Identification of granulocyte CTAP activity. The potential of granulocytes to stimulate fibroblasts ("activation") was compared with that of platelets and mononuclear cells which would serve as sources of previously

Table 1. Stimulation of HA synthesis by leukocyte and platelet extracts*

	Culture HA, μg HA/mg cell protein per 24 hrs	Leukocytes $\times 10^{-7}$/ml extract	P
Experiment 1			
Saline control	8.52 ± 0.59	—	—
Granulocytes	18.46 ± 1.06	1.2	<0.01
Mononuclears and platelets	52.97 ± 0.47	1.1	<0.001
Experiment 2			
Granulocytes	21.00 ± 0.13	1.4	<0.002
Mononuclears and platelets	46.04 ± 0.27	1.1	<0.001

* Pellets of purified granulocytes or mononuclear cells and platelets from Ficoll-diatrizoate gradients were subjected to three rapid freeze-thaw cycles in assay medium, and aliquots of the suspensions were assayed.

identified CTAP factors. Crude cell extracts of the granulocyte and the mononuclear-platelet layers of two Ficoll-diatrizoate gradients were prepared by freeze-thawing the cells in serum-free fibroblast culture medium (Table 1). Seventy-eight percent of the whole blood granulocytes was recovered; mononuclear cell recovery was 50% and platelet recovery was greater than 90%.

The extracts were added to fibroblast cultures, and culture hyaluronate production was measured as an indicator of the activation phenomenon. The granulocyte extracts stimulated significant increases in culture HA ($P < 0.01$, $t = 11.5$). Mononuclear and platelet extracts were much more potent stimulants of HA production, which suggested that most of the CTAP activity in whole blood resides in these cells. These data also indicated that the contamination of granulocyte preparations by relatively few platelets or mononuclear cells might lead to falsely elevated estimates of granulocyte CTAP activity.

In early studies, evaluation of platelet and mononuclear cell contamination in granulocyte preparations were routinely obtained with 1.7 ± 1.0% (SD) mononuclear cell contamination. Platelet contamination was difficult to evaluate with this technique because of platelet and leukocyte clumping. Platelet counts performed under phase contrast optics were more useful. Platelet contamination as estimated by this technique ranged from 5.3 to 51 platelets per 100 granulocytes.

Beta-thromboglobulin was recently described by Pepper and Moore as a platelet-specific protein (15). In synovial fibroblast cultures it has virtually no capacity to stimulate GAG or DNA synthesis (3). The amino acid sequences of β-TG and CTAP-III have recently been reported (16,17). CTAP-III contains four additional amino acids at its N-terminus but is otherwise identical to β-TG. Several antisera prepared in rabbits and in mice with highly purified CTAP-III cross-reacted with samples of β-TG, suggesting that these additional residues do not contribute to the molecules' antigenicity (3). A commercially available radioimmunoassay (Amersham, Chicago) developed for β-TG, but cross-reacting with and also measuring CTAP-III, was used to detect low levels of platelet contamination (18).

Platelets were collected from healthy donors in the presence of EDTA and theophylline and with rapid cooling to prevent loss of platelet contents. The β-TG antigen (β-TG^{Ag}) content of extracts prepared by freeze-thawing platelets in the presence of 0.01% Triton X-100 was measured by radioimmunoassay. These extracts contained 12.5 ± 4.0 picograms β-TG^{Ag} per 10^3 platelets (n = 11). The addition of human thrombin to platelets released 87% of their β-TG^{Ag} during a 10-minute incubation at 21°C.

Aliquots of granulocytes purified on isopycnic Ficoll-diatrizoate gradients were similarly extracted and assayed for β-TG^{Ag} content. Between 0.04 and 0.40 picograms β-TG^{Ag} were present per 100 granulocytes, with a mean of 0.19 ± 11 pg/100 WBC (n = 12). This provided an independent measure of platelet contamination of granulocyte preparations. An average of 15 platelets per 100 granulocytes remained (based on β-TG^{Ag} calculations) in carefully prepared granulocyte pellets. Incubation of aliquots of freshly separated granulocytes with thrombin produced a 77% reduction in their β-TG^{Ag} content as platelet release occurred. This suggested that although small amounts of β-TG^{Ag} might be adsorbed to cell pellets, granulocytes do not contain β-TG^{Ag} or CTAP-III.

To demonstrate that CTAP factors derived from contaminant lymphocytes and platelets were present in quantities too small to stimulate fibroblasts, mononuclear cells (90% lymphocytes) collected from separation gradients (96% purity) were treated with thrombin and washed to free them of platelet contamination. Platelets were collected from fresh plasma with only rare contaminant leukocytes. Both platelets and mononuclear cells were extracted with 80% ethanol, 20% 1.25N HCl (acid-ethanol) at 4°C. Extracts of gradient purified granulocytes were prepared in the same manner. After dialysis against phosphate buffered saline, the volumes of the platelet and mononuclear cell extracts were adjusted so that they were assayed at concentrations proportionate to their numbers as contaminants in separated granulocyte preparations. The β-thrombo-

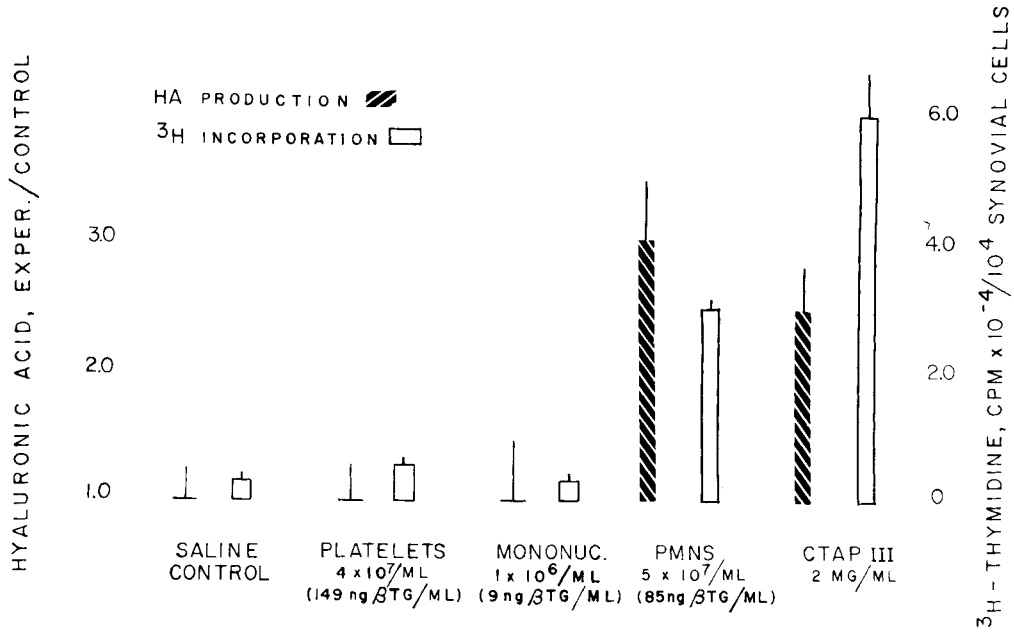

Figure 1. Purified granulocytes or platelets and mononuclear cells in quantities reflecting their presence as contaminants of the granulocytes were extracted with acid-ethanol. Partially purified samples of platelet-derived CTAP-III were used as positive controls. β-Thromboglobulin antigen (**β-TG**) concentrations were determined per ml of extract.

globulin antigen content of the preparations at assay dilution confirmed the accuracy of this adjustment with platelets (Figure 1). Neither the dilute platelet nor the mononuclear cell extract stimulated HA synthesis. The crude granulocyte extract produced substantial increases in both HA production and DNA synthesis. Furthermore, fibroblast DNA synthesis was not significantly increased by the platelet and mononuclear cell extracts at these low concentrations.

These observations suggested that both fibroblast HA and DNA synthesis were stimulated by a granulocyte-derived factor. Several extracts prepared from granulocytes obtained from 5 healthy adults and tested with one strain of synovial fibroblast produced similar levels of stimulation of HA synthesis (Table 2). These extracts also stimulated substantial increases in ^{3}H-methylthymidine incorporation. The potency of a particular extract in stimulating HA synthesis was not proportional to its mitogenic activity in this experiment, and it seemed possible that different granulocyte factors might be biologically active in each assay system.

Extraction and fractionation of CTAP-PMN. Crude granulocyte extracts capable of "activating" synovial fibroblasts could be produced by freeze-thawing

Table 2. CTAP-PMN activity of normal granulocytes

Sample*	PMN × 10^{-7} extracted, ml extract	β-TG^{Ag} ng/ml extract†	HA synthesis, exp/con‡	^{3}H incorporation, exp/con‡	Trypsin equivalents, ng/ml§
A	4.0	8.5	2.71	5.24	14
B	2.2	10.5	4.04	4.62	NT
C	4.0	7.0	2.76	4.45	22
D	4.0	40.0	3.14	3.60	11
E	4.0	20.0	2.76	3.22	35

* Crude acid-ethanol extracts of cells from five healthy adults were assayed using one strain of synovial fibroblasts.

† Platelet contamination was measured by using a radioimmunoassay for the β-thromboglobulin antigen (β-TG^{Ag}).

‡ exp/con = experimental/control.

§ Neutral protease activity was determined using trypsin as a standard. NT = not tested.

Table 3. SP-Sephadex fractionation of acid-ethanol granulocyte extract*

	Fraction protein, μg/ml†	β-TG^{Ag} ng/ml‡	DNA synthesis exp/con ± SD§	HA synthesis exp/con ± SD§
Crude	2,160	85	12.65 ± 0.23	4.81 ± 0.28
RT	850	47	13.45 ± 0.87	4.16 ± 0.20
pH 5.2	727	207	11.19 ± 1.61	3.48 ± 0.29
pH 7.2	594	43	3.73 ± 0.86	2.09 ± 0.26
NaOH	806	—	1.43 ± 0.52	1.63 ± 0.41

* A crude granulocyte extract was applied to SP-Sephadex in 0.05*M* citrate buffer, pH 3.2, and the unbound protein was collected (RT). Elution followed with 0.05*M* citrate + 0.5*M* NaCl, pH 5.2; 0.05*M* citrate + 1.0*M* NaCl, pH 7.2; and 0.1*N* NaOH.

† Fractions were lyophilized and dialyzed versus buffered saline prior to assay.

‡ β-thromboglobulin antigen (β-TG^{Ag}) was measured by radioimmunoassay.

§ exp/con = experiment/control.

in CMRL-1066 culture medium or a balanced salt solution (Hank's), but were of generally low potency and very labile. Extracts prepared in acidic vehicles (pH less than 3) such as 0.02*M* acetic acid or 20% 1.25*M* HCl + 80% ethanol (acid ethanol) were more active. Most of the hyaluronate-stimulating activity of the acid ethanol extracts was precipitated by addition of 3 volumes of cold (4°C) acetone, and could be redissolved in 0.1–1.0*M* citric or acetic acid. The initial acid-ethanol extraction solubilized about 60% of the protein in granulocyte pellets. Approximately 15% of the extracted protein was precipitated when these extracts were dialyzed to pH 7.4 prior to bioassay. Further precipitate formation and loss of biologic potency were often observed in crude preparations stored at −40°C.

Acid ethanol granulocyte extracts were partially purified by cation exchange chromatography on SP-Sephadex. A crude extract was applied to the cation exchange column in 0.05*M* citrate buffer, pH 3.2. Elution with citrate buffer (pH 5.2 containing 0.5*M* NaCl) followed by lyophilization and dialysis versus buffered saline recovered a CTAP-PMN fraction containing about 30% of the bound protein (Table 3). This fraction effectively stimulated HA and DNA synthesis in bioassay. The β-TG^{Ag} concentration of this fraction was enriched in comparison to crude extracts, suggesting copurification of CTAP-PMN, β-TG, and CTAP-III. On SDS slab gel electrophoresis in 15% acrylamide gels, the unbound fraction was similar in appearance to the crude extract (Figure 2). The pH 5.2 fraction contained three distinct bands corresponding to molecular weights between 12,700 and 15,700 daltons. It should be noted that CTAP-III (MW 9,300 daltons) and β-thromboglobulin (MW 8,851 daltons) could not be resolved on these gels. Further evidence regarding the molecular weight of CTAP-PMN was obtained by gel filtration chromatography on Sephadex G-50 or G-75 in 0.2–1.0*M* acetic or citric acid. Fractions which stimulated HA and DNA synthesis were eluted just before and with the elution volume of lysozyme (MW 14,400 daltons).

Characteristics of CTAP-PMN. The partially purified CTAP-PMN preparation eluted as the pH 5.2 SP-Sephadex fraction was used in further characterization studies. The molecular weight of the active factor(s) was estimated to be 14,100 daltons. This preparation produced half-maximal stimulation of ^{3}H-thymidine incorporation at approximately the same concentration (1.0 n*M*/ml) as CTAP-III (Figure 2). This represented a conservative estimate of the biologic potency of CTAP-PMN, since it assumed that all peptides

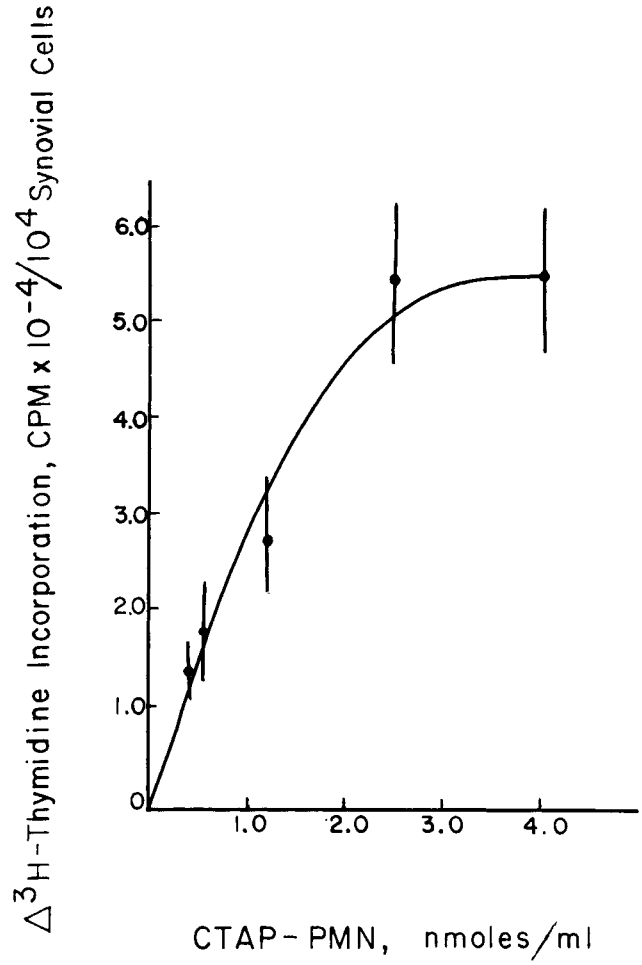

Figure 2. Stimulation of ^{3}H-thymidine incorporation in one strain of human synovial fibroblasts by partially purified CTAP-PMN (pH 5.2 fraction).

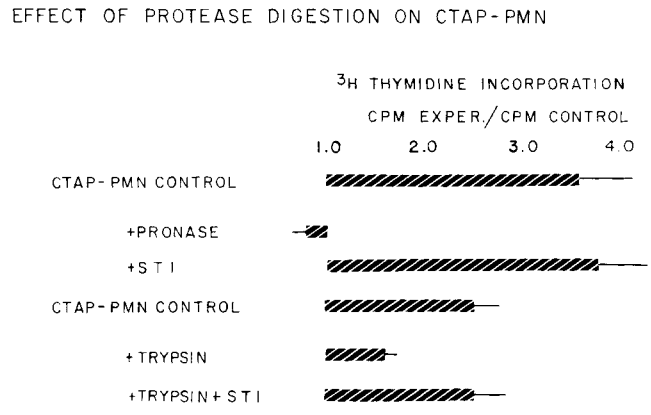

Figure 3. Partially purified CTAP-PMN (pH 5.2 fraction, 3.4 μg) was incubated (37°C, 18 hours) with saline (**control**), **pronase** 0.25 μg, soybean trypsin inhibitor (**STI**) (2.5 μg), **trypsin** 0.25 μg, or **trypsin + STI** 0.25 + 2.5 μg. The mixtures were then assayed.

present were biologically active. This fraction was used to investigate the heat and thiol reagent stability of CTAP-PMN. A 28% loss in mitogenic activity was observed after overnight incubation at 37°C, possibly as a result of autolysis. Forty-four percent of the initial activity remained after exposure to 100°C for 10 minutes followed by cooling at room temperature. A 2-hour exposure to 100°C rendered CTAP-PMN inactive. It did not stimulate ^{3}H-thymidine incorporation after it had been incubated with 0.001*M* dithioerythritol (DTE) at 37°C for 18 hours.

Like the other CTAP factors, CTAP-PMN was inactivated by protease digestion. Trypsin, pronase, and soybean trypsin inhibitors were not mitogenic when added to cultures alone. Incubation of the granulocyte factor with pronase prior to adding the mixture to fibroblast cultures abolished the mitogenic potential of the factor (Figure 2). Trypsin was less destructive than pronase at 50 μg/ml concentrations. When proteolytic digestion of CTAP-PMN by trypsin was prevented by including an excess of soybean trypsin inhibitor in the incubation mixture, CTAP-PMN activity was preserved.

Crude CTAP-PMN preparations contained protease activity against a hide powder substrate at pH 7.0 equivalent to between 11 and 35 ng of trypsin/ml. The protease activity of these extracts did not appear to correlate with their effectiveness as stimulants of HA or DNA synthesis (Table 2). Furthermore, crude CTAP-PMN effectively stimulated fibroblast DNA synthesis in the presence of soybean trypsin inhibitor at a concentration (500 μg/ml) which blocked hide powder digestion.

Partially purified preparations of CTAP-PMN were tested in Ouchterlony plates with antisera to CTAP-III raised in rabbits and mice. No precipitin lines were formed with CTAP-PMN preparations.

DISCUSSION

Identification of a specific granulocyte CTAP factor required highly purified granulocytes. Using isotonic density gradients, Loos and Roos determined that granulocytes have a buoyant density of 1.082 gm/ml (19). An upper limit for the buoyant density of normal platelets was determined by Corash et al to be 1.084 gm/ml, with only 14% of the platelet population being more dense than 1.071 gm/ml (20). Accordingly, Ficoll-diatrizoate gradient mixtures designed to recover lymphocytes at a specific gravity of 1.077 gm/ml allow significant numbers of platelets to pass through the gradient interface and mix with the more dense granulocytes and erythrocytes.

By using Ficoll-diatrizoate gradients similar to those devised by Böyum, but made nearly isopyric for granulocytes (1.083 gm/ml), we retained 99.2% of the platelets in whole blood above the gradient interface and recovered purified granulocytes below this interface (8).

A radioimmunoassay for the platelet β-thromboglobulin antigen was used to measure low levels of platelet contamination in separated granulocytes. Approximately 400 platelets/mm^3 could be detected with this assay, corresponding to 0.2% of the normal blood platelet count. Normal platelets were found to contain 12.5 ± 4.0 pg β-TG^{Ag} per 10^3 platelets. By using a mean normal platelet density of 1.058 gm/ml (19) and a mean platelet volume of 5.17 μm^3 (20), it was calculated that 2.55 ± 0.82 mg β-TG^{Ag} was present in a gram of fresh platelets. Pepper and Moore have reported a similar value of 1.24 mg β-thromboglobulin per gram of platelets (15). Their somewhat lower value might reflect the facility with which β-TG is released from platelets in vitro.

We have applied the term "CTAP-PMN" to the granulocyte-derived factor which stimulates both DNA and HA synthesis in synovial fibroblasts. It was extracted from granulocytes under essentially the same conditions used in preparing CTAP-III from platelets, i.e., low pH and low ionic strength.

Since measurements of the β-thromboglobulin antigen by radiommunoassay did not discriminate between mitogenic CTAP-III and inactive β-thromboglobulin, the CTAP-III content of CTAP-PMN prepa-

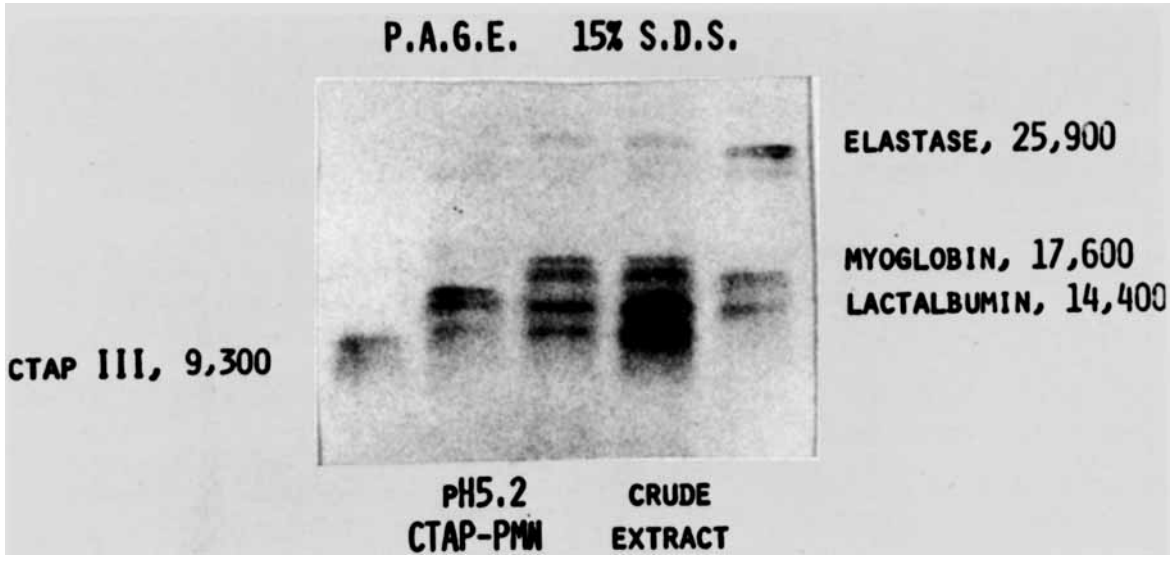

Figure 4. Slab gel electrophoresis using SDS CTAP-PMN activity resides in a protein(s) with a molecular weight between 12,000–16,000 daltons.

rations was estimated. The dose response curve of purified CTAP-III suggests that greater than 500 ng/ml of that factor is required to stimulate two-fold or greater increases in culture ^{3}H-thymidine incorporation (3). No more than 207 ng/ml β-TG^{As} were present in any of the CTAP-PMN preparations assayed, evidence that the observed stimulation of DNA synthesis by these extracts was not attributable to CTAP-III contamination.

In crude CTAP-PMN extracts, small quantities of other platelet growth factors may have been present. However, the few platelets contaminating these preparations did not stimulate increases in DNA synthesis when they were assayed alone (Figure 1). The possibility that CTAP-PMN activity may be additive to or potentiate platelet growth factor activity has not yet been investigated.

Partially purified CTAP-PMN contained at least three peptides with molecular weights ranging between 12,700 and 15,700 daltons. Current evidence suggests that one peptide stimulates both hyaluronate and DNA synthesis. It was not possible to diminish one biologic activity with heat, thiol reagents, or by proteolytic digestion without concomitant loss of the other. Like platelet-derived growth factor(s), CTAP-PMN was relatively resistant to heating at 100°C. Most of the currently identified mitogenic factors active on connective tissue contain intramolecular disulfide bonds which are required for their biologic activity (6). CTAP-PMN was inactivated by incubation with dithioerythritol, suggesting that it contains at least one disulfide bond. Like the previously described CTAP factors, its biologic activity was protease-labile. Important antigenic differences appear to exist between CTAP-III and CTAP-PMN, since precipitin lines failed to form between anti-CTAP-III antibody and CTAP-PMN preparations in Ouchterlony analysis.

The hypothesis that weak neutral protease activity present in preparations of CTAP-PMN was responsible for the observed stimulation of ^{3}H-thymidine incorporation was investigated by using soybean trypsin inhibitor. Although the activity of CTAP-PMN was not diminished by the presence of this protease inhibitor, the role that granulocyte proteases may play in synovial fibroblast activation and growth has not been determined.

In 1921, Carrel applied the term "trephone" to the growth-promoting substances presumably present in inflammatory cells (22). Investigators of wound healing have generally related the extent of fibrosis in healed experimental wounds to the intensity of inflammatory response (23). The studies of Simpson and Ross suggested that neutrophils were not required for wound healing in guinea pigs treated with antineutrophil serum, since neutropenic and control wounds contained histologically similar numbers of fibroblasts and collagen fibers (24). On the other hand, extracts of guinea pig leukocyte lysosomes were reported to simulate proline incorporation in fibroblast cultures and to increase the mechanical resistance of healing skin wounds (25).

The in vivo importance of CTAP-PMN as a

mitogenic factor in wounds, in the joint space, or at other sites of acute and chronic inflammation remains unclear. The liberation of chemotactic factors at sites of tissue injury quickly attracts large numbers of granulocytes from the vascular space. Platelets appear to be more potent than the granulocytes as a source of factors which promote fibroblast "activation" and DNA synthesis in vitro. However, there is little evidence to suggest that platelet release continues to occur at sites of injury after hemostasis has been established. Granulocyte and monocyte-derived factors might be expected to play major roles in modulating the later cellular responses to injury. The generally sequential recruitment of platelets, granulocytes, macrophages, and lymphocytes in the response to tissue injury might permit CTAP factors from each cell type to participate in the local control of fibroblast synthetic activity.

REFERENCES

1. Castor CW: Synovial cell activation induced by a polypeptide mediator. Ann NY Acad Sci 256:304–317, 1975
2. Castor CW, Ritchie JC, Scott ME, Whitney SL: Connective tissue activation. XI. Stimulation of glycosaminoglycan and DNA formation by a platelet factor. Arthritis Rheum 20:859–868, 1977
3. Castor CW, Ritchie JC, Williams CH Jr, Scott ME, Whitney SL, Myers SL, Sloan TB, Anderson BE: Connective tissue activation. XIV. Composition and actions of a human platelet autacoid mediator. Arthritis Rheum 22:260–272, 1979
4. Koyama H, Tomida M, Ono T: The enhanced production of hyaluronic acid by cultured rat fibroblast cells treated with cyclic AMP and its dibutyryl derivative. J Cell Physiol 87:189–197, 1975
5. Tomida M, Koyama H, Ono T: A serum factor capable of stimulating hyaluronic acid synthesis in cultured rat fibroblasts. J Cell Physiol 91:323–328, 1977
6. Castor CW: Regulation of connective tissue metabolism, Arthritis and Allied Conditions. Ninth edition. Edited by DJ McCarty. Philadelphia, Lea & Febiger, 1979, pp 201–213
7. Yaron M, Castor CW: Leukocyte-connective tissue cell interaction. I. Stimulation of hyaluronate synthesis by live and dead leukocytes. Arthritis Rheum 12:365–373, 1969
8. Böyum A: Separation of leukocytes from blood and bone marrow: introduction. Scand J Clin Lab Invest 21(97):7, 1968
9. Cutts JH: Cell separation—methods in hematology. New York, Academic Press, 1970
10. Castor CW: Regulation of collagen and hyaluronate formation in human synovial fibroblast cultures. J Lab Clin Med 75:798–810, 1970
11. Castor CW: Connective tissue activation. I. The nature, specificity, measurement and distribution of connective tissue activating peptide. Arthritis Rheum 14:41–54, 1971
12. Bitter T, Muir HM: A modified uronic acid carbazole reaction. Anal Biochem 4:330–334, 1962
13. Oyama VI, Eagle H: Measurement of cell growth in tissue culture with a phenol reagent (Folin-Ciocalteau). Proc Soc Exp Biol Med 91:305–307, 1956
14. Rindeknecht H, Geokas MC, Silverman P, Haverback B: A new ultrasensitive method for the determination of proteolytic activity. Clin Chim Acta 21:197–203, 1968
15. Ludlam CA, Anderton JL: Platelet β-thromboglobulin, Proceedings from Workshop on Platelets. Edited by HJ Day, H Holmsen, MB Zucker. DHEW, NIH 78-1087, 1978, pp 267–290.
16. Begg G, Pepper D, Chesterman C, Morgan F: Complete covalent structure of human β-thromboglobulin. Biochemistry 17(9):1739–1744, 1978
17. Walz DA, Castor CW: Connective tissue activation: structural studies on a human platelet mitogen. Clin Res 27(4):649, 1979
18. Myers S, Castor CW, Anderson BE: Measurement of CTAP-III levels by RIA in normal and rheumatoid plasma. Clin Res 27(4):646, 1979
19. Loos JA, Roos D: Ficoll-isopaque gradients for the determination of density distributions of human blood lymphocytes and other reticulo-endothelial cells. Exp Cell Res 86:333–341, 1974
20. Corash L, Tan H, Gralnick H: Heterogentity of human whole blood platelet subpopulations. I. Relationship between buoyant density, cell volume and ultrastructure. Blood 49(1):71–87, 1977
21. Ludlam CA, Cash JD: Studies on liberation of β-thrombogloublin from human platelets in vitro. Br J Haematol 33(2):239–247, 1976
22. Carell A: Cicatrization of wounds. XII. Factors initiating regeneration. J Exp Med 34:425–434, 1921
23. Simpson DM, Ross R: The neutrophilic leukocyte in wound repair. J Clin Invest 51:2009–2023, 1972
24. Selye H: Part of inflammation in local adaptation syndrome, International Symposium on the Mechanism of Inflammation. Edited by G Jasmin, A Robert. Montreal, Acta Inc., 1953, p 53
25. Tchórzewski H, Warno O, Szram S, Szumega A, Niedworok J, Andrzejewski W, Sulowska Z: The effect of lysosomal proteins of granulocytes and macrophages on healing of surgical wounds in guinea pigs. Acta Med Pol 17(1):41–54, 1976