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Connective Tissue Activation

XVI. Detection of a Human Platelet-Derived Connective Tissue Activating Peptide (CTAP-III) in Human Sera and Plasma and in Synovial Fluids and Tissues (40860)

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Abstract. Antisera were prepared to the platelet-derived connective tissue activating peptide (CTAP-III) and used to ascertain the presence of CTAP-III materials in a number of biologic specimens by double immunodiffusion analyses. Materials reactive with anti-CTAP-III sera were found in all human sera and platelet extracts tested; weak reactivities were obtained in human plasma specimens but no reactivity was seen in plasma samples for which care was taken to avoid platelet activation. Extracts of synovial fluids and tissues and a spleen extract, processed in the manner similar to the CTAP-III purification scheme from platelet extracts, exhibited positive reactivities. However, synovial fluids contained no detectable reactivity by the double immunodiffusion method. The anti-CTAP-III sera were not reactive with the many polypeptide hormones and growth factors tested, including the CTAP-I and -II factors and platelet factor-4. Previous studies had shown that CTAP-III, β-thromboglobulin, and the low affinity platelet factor-4 share similar or identical antigenic determinatants. CTAP-III has mitogenic activity for many strains of human connective tissue derived cells and the presence of CTAP-III reactive components in synovial tissues suggests that CTAP-III may in part contribute to the proliferative response of those tissues during inflammation.

The *c*onnective *t*issue *a*ctivating *p*eptides (CTAP) derived from lymphocytes and lymphoid tissues (CTAP-I) (1–5), HEp-2 cells (CTAP-II) (6), and platelets (CTAP-III) (5, 7–9) have been shown to induce a number of metabolic changes in cultured human connective tissue cells. These include the stimulation of glycosaminoglycan synthesis, glucose consumption, and lactate formation. In addition, the CTAP-III (from platelets) has been shown to be a potent mitogen for 17 strains of human synovial, dermal, cartilage, and thyroid connective tissue cells (7, 10).

The platelet-derived CTAP-III was shown to be a major mitogenic component of human serum (9, 10). CTAP-III has been purified to homogeneity and its composition and biological activities described (7–12). Recently, CTAP-III has been shown to be immunologically identical to the low affinity platelet factor-4 ($LAPF_4$) (10), and to β-thromboglobulin (βTG) (13), factors found subsequent to platelet release reactions. The studies reported here show that antisera can be readily produced to CTAP-III and that the antisera can be used to evaluate the presence of CTAP-III in a variety of biologic preparations. CTAP-III was demonstrated in human and monkey sera, human plasma, synovial fluid and tissue preparations, and in a human spleen preparation. The possible involvement of CTAP-III in the potentiation of tissue inflammation, particularly synovial tissue, is discussed.

Materials and methods. CTAP-III was purified from human platelets as previously described (7, 9, 10) and was homogeneous by the criteria of giving single protein bands in polyacrylamide gel electrophoresis in SDS and acid–urea buffer systems. Antisera to CTAP-III were produced by immunizing two 5-lb white New Zealand male rabbits bi-weekly using multiple intramuscular, subcutaneous, and intraperitoneal

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injections of CTAP-III emulsified in adjuvants. The first injections included giving CTAP-III emulsion in the foot pads and an intravenous injection of 50 μg of CTAP-III in aqueous solution. The emulsions used for the first four injections consisted of 200 μg of CTAP-III in complete Freund's adjuvant with Maalox (1:1). The third and fourth injections utilized CTAP-III (200 μg) fixed with 1% glutaraldehyde and 100 μg of CTAP-III treated by heating at 100° for 1 min, respectively. All subsequent multiple injections done at monthly intervals utilized 100 μg of CTAP-III in phosphate-buffered saline (PBS), pH 7.2, emulsified in incomplete Freund's adjuvant (1:1). The animals were bled (25–30 ml) every 7–10 days and sufficient iron–dextran (Hyferdex, Elanco) was given intramuscularly to maintain a normal hematocrit.

Double immunodiffusion analyses of CTAP-III and potential sources of CTAP-III against anti-CTAP-III sera were performed using Immuno-tec agarose plates (Behring Diagnostics). To improve detection and to enhance precipitin bands, some double immunodiffusion analyses utilized a second antibody technique. After the initial antiserum and antigen sources had been allowed to diffuse and react for 24 to 48 hr, the immunodiffusion plate was washed extensively with PBS (containing 0.5% NaN_3, PBS–azide) at 37° for 2 days. The agarose plates were then overlaid with the immunoglobulin (Ig) fraction of goat anti-rabbit Ig (Cappel Labs, antibody titer equal to approximately 2 mg/ml) and incubated at 22° for 24 hr. Some of the plates were then washed with PBS–azide and stained with triple stain (14). In the extensive number of immunodiffusion analyses performed in this study, it was ascertained that the second antibody procedure did not result in any additional precipitin band formation other than that between the CTAP-III protein and antibodies of the anti-CTAP-III sera. Furthermore, when commercial antisera or antisera prepared in these laboratories to purified human plasma components other than CTAP-III were tested by immunodiffusion and the second antibody technique to CTAP-III, no precipitin band formation was noted. The sensitivity of the second antibody procedure was estimated by using various dilutions of purified CTAP-III alone and added to a synovial fluid which was negative for precipitin band formation by these analyses. The second antibody technique improved detection of precipitin band formation approximately fivefold. CTAP-III at a concentration of 2 μg/ml (or 30 ng per 15 μl of sample applied in the antigen wells) resulted in noticeable precipitin formation. Immunoelectrophoretic analyses were done as previously described (15) and the plates processed and stored in a manner similar to the immunodiffusion plates.

Human sera, plasma, urine, and saliva were collected from volunteers. Cord sera, synovial fluids, cerebrospinal fluid, pleural fluids, and kidney cyst fluid were portions of samples that had been collected for diagnostic purposes. Mouse, rat, guinea pig, and chicken sera and rhesus monkey serum and plasma were taken from laboratory animals, and horse, bovine, fetal calf, lamb, goat, and porcine sera were commercial preparations. The human spleen specimen was obtained at autopsy. Synovial tissues were obtained from patients undergoing joint surgery.

Four of the synovial tissue specimens and the spleen material (1–5 g), a sample of human serum (5 ml), and one RA and one osteoarthritic synovial fluid (5 ml of each) were processed in a manner similar to the preparation of purified CTAP-III (7, 9, 10). The tissues were minced and washed with PBS and then extracted with acid–ethanol (5% 1.25 *N* HCl:95% EtOH) for 1 hr at room temperature. Insoluble material was removed by centrifugation, material soluble in acid–ethanol precipitated with three volumes of acetone, the precipitate dissolved in 1.0 *M* acetic acid, and dialyzed against PBS. The retentate was centrifuged and the supernatant used in immunodiffusion analyses after concentration. Some of the preparations were further fractionated on a Sephacryl S-200 (Pharmacia) column equilibrated and eluted with PBS. Material eluting in the position of cytochrome-c was concentrated and tested for CTAP-III by immunodiffusion.

Three synovial tissues of rheumatoid arthritic (RA) origin were homogenized in

PBS (1 g of tissue per 4 ml of PBS) using a Tekmar tissue homogenizer and the solutions centrifuged at 1500*g* for 30 min. The particulate material was extracted with 0.2 *M* glycine–HCl, pH 2.2 (4 ml per gram of tissue), and the extract dialyzed against PBS. The original supernatant and the glycine extracts were tested by immunodiffusion. Synovial fluids were also tested directly for CTAP-III by the immunodiffusion analyses.

Many of the synovial fluids had been collected, used for various other diagnostic and experimental purposes, and stored over a long period of time at −20° or −90°, other synovial fluids were obtained concurrent with these studies and tested for CTAP-III soon after collection.

Platelets were isolated from normal human plasma by differential centrifugation, red and white blood cells being removed at 100*g*, and platelets collected in the 1500*g* pellet. An extract was prepared by freeze–thawing three times 0.1 ml of packed platelets in 1 ml of 0.016 *M* Tris–HCl containing 0.85% NaCl and 0.006 *M* dextrose, pH 7.35, with or without the protease inhibitors aprotinin (100 Kallikrein inhibitor units/ml), ϵ-amino caproic acid (2.5 m*M*), *p*-hydroxymercuribenzoate (1.3 m*M*), and pepstatin (0.15 m*M*). The protease inhibitors were purchased from Sigma Chem. Company. Three plasma samples were collected using Na_2EDTA as anticoagulant. One blood sample was drawn into a glass syringe and the plasma taken as the supernatant from centrifugation at 1500*g* for 30 min. Blood samples were also drawn from two additional volunteers into two consecutive vacutainer tubes and the plasma obtained after centrifugation at 1500*g* for 30 min was tested for immunoreactive CTAP-III. Platelet-poor plasma was prepared by drawing blood in a plastic syringe after discarding the first 3 ml. The blood was divided into three portions and one-tenth volume was added of a mixture consisting of 0.13 *M* Na citrate plus 2% dextrose, 1% Na_2EDTA plus 0.7% NaCl, or Na heparin (Panheparin, Abbott Lab, 10,000 units/ml). The blood fractions were centrifuged at 100 *g* for 15 min at 22° in polystyrene tubes and the plasma removed and centrifuged at 1500*g* for 30 min. The top half of the platelet-poor plasma was removed and used for testing against anti-CTAP-III sera.

Saliva and urine samples were obtained from volunteers, the saliva being used directly in immunodiffusion analyses against anti-CTAP-III. The urine samples were collected as the first morning urine. These were lyophilized, dissolved in 1/20th the original volume with water, dialyzed for 3 days against distilled water in Spectrapor tubing (Spectrum Medical Industries, M_r exclusion of 2000 M_r), and tested for the presence of CTAP-III reactive components.

Polypeptides tested for possible identity or cross-reactivity to anti-CTAP-III serum were obtained from commerical sources; the numbers refer to the amounts, in micrograms, used in the double immunodiffusion analyses; human chorionic gonadotropin, 20; human gastrin, 20; bovine glucagon, 20, and insulin, 90; bovine thyroid stimulating hormone, 20, and parathyroid hormone, 20; somatostatin, 20; bradykinin, 20; adrenal corticotropic hormone, 40; human prolactin, 0.5; synthetic porcine luteinizing releasing hormone, 20; nerve growth factor, 20 (obtained from Dr. J. Tomita); and bovine fibroblast growth factor, 1, and mouse epidermal growth factor, 10 (both from Collaborative Research). Purified preparations of CTAP-I and CTAP-II were prepared as previously described (6); the high affinity platelet factor 4 (PF_4) was a gift from Dr. S. N. Niewiarowski and antiserum to PF_4 was kindly supplied by Dr. M. H. Ginsberg.

Results. An antibody response to CTAP-III was detected in the immunized rabbits as early as 2 weeks from the initial antigen injections using the second antibody immunodiffusion technique. By 9 weeks postimmunization the titers of the antisera were sufficient to detect precipitin bands with CTAP-III without the second antibody. The rabbits continued to produce substantial anti-CTAP-III throughout the monthly booster injections. Antisera from the 12th through 27th week were used for detection of CTAP-III in the various sources utilized. Some of the antisera

showed a weak precipitin band formation against human albumin. That antibody specificity was removed by absorption of those antisera with glutaraldehyde-polymerized albumin.

Immunoreactive CTAP-III was demonstrable in a number of specimens utilizing double immunodiffusion, with or without application of a second anti-Ig to enhance precipitin band formation. The various fluids and tissues are listed in Table I. All human sera, regardless of source, were positive by precipitin band formation for CTAP-III. The plasma samples were much less reactive than sera. When blood was drawn into two consecutive vacutainers using Na_2EDTA as anticoagulant, the plasma from the first vacutainers were weakly reactive as discerned through use of the second antibody technique whereas the plasma in the second vacutainer were even less reactive. Plasma obtained from blood

TABLE I. SPECIMENS CONTAINING ANTIGENS REACTIVE WITH ANTI-CTAP-III SERA[a]

Specimens	Number positive/number tested
Human sera	
Adult[b]	21/21
Cord sera	5/5
A-E extract[c]	1/1
A-E, S-200[c]	1/1
Human plasma[d]	3/3
Platelet-poor plasma[e]	1/3
Platelet extracts[f]	All positive
Synovial fluids[g]	0/50
A-E extracts, S-200[h]	2/2
RA synovial tissues	
PBS extracts	0/3
0.2 *M* glycine–HCl extracts	0/3
A-E extracts, S-200	4/4
Spleen	
A-E extract	1/1
A-E, S-200	1/1
Urine samples	0/4
Amniotic fluids	0/3
Saliva	0/3
Kidney cyst and cerebrospinal fluids	0/2

[a] Specimens were tested for immunoreactive components by double immunodiffusion analyses in agarose gels as described in the text. The specimens designated as negative were those that yielded no discernible precipitin band even after application of the second antibody technique and staining of the gels.

[b] Included sera from 10 individuals with various types of arthritis.

[c] Acid–ethanol extract and Sephracryl-200 procedures were used as described in the methods section.

[d] Two of the plasma samples were obtained by drawing blood into two consecutive vacutainer tubes. The plasma from both vacutainers were weakly reactive after using the second antibody method for detection of precipitin band formation; the plasma from the second vacutainer was less reactive.

[e] Three portions of the platelet-poor plasma were obtained from each blood sample as detailed in the text. Only one of the portions was positive by a weak precipitin band formation which was noted after use of the second antibody technique and staining of the gel.

[f] Many platelet extracts have been prepared and all have been positive. In addition, a strong precipitin band was obtained from a platelet extract prepared both in the presence and absence of protease inhibitors (see Fig. 1).

[g] Of the 50 synovial fluids, 22 were tested soon after collection and 28 had been stored frozen for greater than 18 months; 28 of the synovial fluids were from individuals with RA and 22 from individuals with a variety of other arthritic diseases including traumatic arthritis, osteoarthritis, gout, and systemic lupus erythematosus.

[h] One RA and one osteoarthritic synovial fluid were processed by acid–ethanol extraction and Sephracryl-200 chromatography and concentration as described in the text.

drawn into a glass syringe was weakly reactive. All the portions of the platelet-poor plasma, with the exception of one in which Na_2EDTA was used as anticoagulant, were unreactive even after the second antibody and staining procedures.

In addition to human sera, sera from several species were evaluated for reactivity with anti-CTAP-III serum. Of those tested, only the rhesus monkey serum resulted in a strong precipitin band; the monkey plasma from blood drawn into a glass syringe with Na_2EDTA as anticoagulant was weakly reactive. All other animal sera gave no detectable precipitin bands even after application of the second antibody procedure. None of the large number of synovial fluids tested, even those tested in double immunodiffusion soon after collection were reactive with anti-CTAP-III serum. Likewise, the saliva, amniotic fluids, urine samples, kidney cyst, and cerebrospinal fluids were unreactive.

Three RA synovial tissues were homogenized in PBS, and both the PBS supernatants and the glycine–HCl extracts of the particulate material were not reactive with anti-CTAP-III serum. However, in order to concentrate CTAP-III in the tissues and to ascertain whether the antigenic material in the various sources would fractionate in a similar manner to CTAP-III, four RA synovial tissues were extracted with acid–ethanol and chromatographed on Sephacryl-200 as described under Methods. The materials eluting in the region of cytochrome-c were all positive by reactivity with anti-CTAP-III serum. A human serum, the spleen specimen, and two synovial fluids processed in the same way were also positive in the corresponding gel filtration fractions.

The nonreactivity of many specimens could have been due to rapid degradation of any CTAP-III present. In order to test this possibility, purified CTAP-III was added to freshly drawn synovial fluids (one traumatic arthritis and one RA), the fluids incubated at 37°, and aliquots removed at intervals up to 48 hr tested by double immunodiffusion. A fresh human serum sample was also tested in the same way. Although there was some diminution of the CTAP-III precipitin bands in the samples by 48 hr, substantial CTAP-III reactivity was still present.

Figure 1 shows the reaction of anti-CTAP-III serum with purified CTAP-III, a platelet extract prepared by freezing and thawing (with and without protease inhibitors), human serum, and human plasma obtained with care to avoid platelet activation. The precipitin band formed from purified CTAP-III fused with that of the other antigen sources and there was little or no reactivity with the plasma. In all the immunodiffusion precipitin patterns developed, the precipitin band formed between anti-CTAP-III serum and purified CTAP-III fused with the reactive components of the various antigen sources with no apparent spurring. Thus, the antigens in those sources that were reactive were immunochemically similar to CTAP-III. Furthermore, purified CTAP-III and the serum reactive material showed the same mobilities by immunoelectrophoretic analyses and indicated a pI for CTAP-III of approximately 8.6, a value close to that of 8.5 ± 0.4 previously determined by isoelectric focusing (10).

The anti-CTAP-III sera were not reactive with any of the peptide hormones or growth factors listed in the Materials section, and

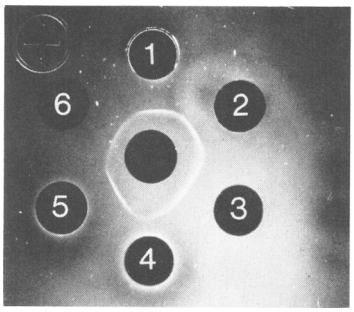

FIG. 1. Double immunodiffusion analysis and application of the second antibody technique were performed as described in the text. The center well contained anti-CTAP-III serum and the antigen wells contained: (1) platelet extract; (2) platelet extract prepared in the presence of protease inhibitors listed under Methods; (3 and 6) purified CTAP-III; (4) human plasma; (5) human serum. A single precipitin band formed with each antigen source, except the human plasma, which fused with the precipitin band formed from the purified CTAP-III.

did not react with purified CTAP-I or -II preparations nor with purified PF_4. Antiserum to PF_4 was also not reactive with purified CTAP-III.

Discussion. These results demonstrate that rabbit antisera can be readily prepared to CTAP-III and used to evaluate the presence of CTAP-III, or antigenically related materials, in a variety of fluids and tissue sources. Utilizing immunodiffusion, reactivities with the antisera could be found in all human sera and some plasma samples. The components in these antigen sources were immunochemically similar to CTAP-III as shown by fusion of precipitin bands with purified CTAP-III and by the immunoelectrophoresis results. Anti-CTAP-III sera reactive components were detected in plasma from blood drawn into a glass syringe and into two consecutive vacutainers, although the precipitin bands formed were considerably weaker than that obtained with serum. Also, there was a lack of plasma reactivity when precautions were exercised to prevent platelet activation (in the preparation of platelet-poor plasma). These results suggest that the plasma and sera reactivities are primarily if not exclusively derived due to release from platelets.

All synovial fluids tested for reactivity with anti-CTAP-III sera were not reactive although two of the fluids processed by acid–ethanol extraction and Sephracryl-200 chromatography were reactive. In a similar manner, the spleen speciman and four synovial tissues processed in the same way were reactive whereas synovial tissues extracted with PBS and glycine–HCl were unreactive. Thus, any CTAP-III in unfractionated synovial fluids may be below the limits of detectability by double immunodiffusion analysis and second antibody application, or other substances may interfer with detection of CTAP-III reactive material, or CTAP-III may be degraded to a large extent if originally present in those specimens. However, addition of purified CTAP-III to synovial fluids (and of the CTAP-III in human serum) showed that degradation of the antigenic determinant at 37° for up to 48 hr was not substantial. Also, the fractionation of synovial fluids and tissues by acid–ethanol and Sephacryl –200 showed that the reactive material was similar to that of purified CTAP-III.

An important role for CTAP-III mediation in atherosclerotic, thrombotic, and inflammatory processes has been suggested (10). It has been shown that individuals with RA may exhibit thrombocytosis and that RA platelet lysates have decreased CTAP-III activities (16). Because CTAP-III is a mitogen for fibroblasts, including the fibroblast-like synovial-derived cells, the presence of CTAP-III in the extracted synovial fluids and RA synovial tissues may be of significance to the proliferative state of the RA synovial tissue. The concentration of CTAP-III which produces the various biologic responses is approximately 10^{-7} *M* (10) which is equal to 1 μg of CTAP-III/ml. The sensitivity of the immunodiffusion analyses with application of the second antibody procedure could detect a concentration of CTAP-III in synovial fluids, serum, and plasma of 2 μg/ml, which is greater than that needed to produce the biological effects. The latter sensitivity is equivalent to detection of about 30 ng of CTAP-III in the 15-μl samples used in the immunodiffusion analyses. The sensitivity of the immunodiffusion–second antibody procedure is much less than that of the radioimmunoassays for similar proteins. For example, Dawes *et al.* (17) report that the radioimmunoassay of the platelet-derived β-thromboglobulin (βTG) gave values of that protein concentration in platelet-poor plasma of 70.7 ± 13.7 ng/ml, concentrations lower than would be detectable by the immunodiffusion techniques. The latter report also shows that the radioimmunoassay is sufficiently sensitive to quantitate βTG in low concentrations in cerebrospinal fluid and urine specimens. Thus, of the many specimens in Table I that were listed as negative, CTAP-III reactive material may be present but at concentrations below the sensitivity of the immunodiffusion–second antibody procedure.

The anti-CTAP-III sera were specific for CTAP-III in that the antisera were unreactive with the many hormones and growth factors tested, including CTAP-I and CTAP-II, and no reactivity was noted with

another platelet release factor, PF_4. Also, anti-PF_4 serum was unreactive with CTAP-III. It has been reported that mouse antisera to CTAP-III is reactive with the low affinity platelet factor 4 ($LAPF_4$) (10) and that anti-$LAPF_4$ serum is reactive and forms precipitin lines of identity with CTAP-III (10, 18).

The physical–chemical and biologic properties of the various platelet-derived factors, βTG, PF_4, $LAPF_4$, and CTAP-III, have been described in several recent reports. βTG has been extensively characterized by Moore *et al.* (19, 20) and by Begg *et al.* (21) and several studies suggest that βTG measurements in platelet-poor plasma may be useful for assessment of *in vivo* platelet function (cf. 22). The complete amino acid sequence of βTG has been determined and the sequence shows about 50% homology with the sequence of PF_4 (21). Walz and Castor (23) and Rucinski *et al.* (13) have shown that the amino-terminal sequences of CTAP-III and $LAPF_4$ are similar to that of βTG. In the work of Walz and Castor (23), residues 5–16 of CTAP-III were the same as residues 1–12 of βTG, CTAP-III had the additional tetrapeptide amino-terminal sequence of Asn–Leu–Ala–Lys, and the last four residues of the carboxyl-terminal sequences of CTAP-III and βTG were the same. Rucinski *et al.* (13) showed that $LAPF_4$ had the same additional tetrapeptide sequence at the amino-terminal end as does CTAP-III and that amino acid residues 5–12 of $LAPF_4$ were the same as residues 1–8 of βTG. All of the laboratories (10, 13, 21) and this report have now confirmed the various immunochemical reactivities of the four proteins: antisera to PF_4 are not reactive with βTG, $LAPF_4$, or CTAP-III, and anti-βTG, -$LAPF_4$, or -CTAP-III sera are not reactive with PF_4 whereas βTG, $LAPF_4$, and CTAP-III react with each of the respective antisera forming precipitin bands of apparent identities. Thus, $LAPF_4$ and CTAP-III may be identical, and βTG may be identical except for the additional tetrapeptide sequence at the amino-terminal ends of CTAP-III and $LAPF_4$. $LAPF_4$ and CTAP-III also have similar biologic activities whereas βTG lacks the mitogenic activity and certain other biologic properties exhibited by CTAP-III (10). Thus, detection of materials in this study with anti-CTAP-III sera may include the $LAPF_4$ and βTG and the presence of the CTAP-III reactive materials may not necessarily correlate with the biologic properties described for CTAP-III.

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