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16. 2-*n*-Propylhexanoic acid (94 ng) and 2-(3-hydroxy-*n*-propyl)-hexanoic acid (72 ng) were added to acidified microsomal incubations as internal standards. Samples were extracted twice with 3 ml of ethyl acetate and the combined organic extracts were concentrated under a stream of dry nitrogen and derivatized with *bis*(trimethylsilyl)trifluoroacetamide. GC-MS analyses were carried out with a Hewlett-Packard model 5970 mass selective detector connected to an HP model 5890A gas chromatograph and interfaced to an HP model 59970C data system. The gas chromatograph was equipped with a fused silica capillary column (60 m by 0.32 mm internal diameter) coated with the bonded stationary phase DB-1 (J & W Scientific, Ventura, CA). Samples were injected in the splitless mode and "cold-trapped" on the column at 40°C, following which the oven temperature was raised rapidly to 80°C. A linear temperature gradient of 2°C per minute was then maintained to 120°C, after which the temperature was raised by 4°C per minute to 200°C. The temperatures of the ion source and the GC interface were maintained at 200° and 250°C, respectively. The mass spectrometer was operated in the selected ion-monitoring mode, and the following ions were used for quantitative analyses: mass-to-charge ratio (*m/z*) 199 (Δ^4-VPA TMS; $[M-CH_3]^+$), 100 (4-OH-VPA γ-lactone; $[M-C_3H_6]^{\cdot+}$), and 289 (3-OH- and 5-OH-VPA *bis*-TMS; $[M-CH_3]^+$). The retention times of the TMS derivatives of the various VPA metabolites were: Δ^4-VPA, 16.2 minutes; 3-OH-VPA, 29.3 minutes; 4-OH-VPA γ-lactone, 15.7 and 16.0 minutes (two diastereoisomers); and 5-OH-VPA, 32.9 minutes. (Δ^4-VPA was synthesized as described by A. W. Rettenmeier *et al.* [*Drug Metab. Dispos.* **13**, 81 (1985)], whereas Δ^3-VPA was obtained as described previously (*10*).
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Substance P Activation of Rheumatoid Synoviocytes: Neural Pathway in Pathogenesis of Arthritis

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Several clinical features are consistent with nervous system involvement in the pathogenesis of rheumatoid arthritis. The neuropeptide substance P is one possible mediator of this interaction, since it can be released into joint tissues from primary sensory nerve fibers. The potential effects of the peptide on rheumatoid synoviocytes were examined. The results show that substance P stimulates prostaglandin E_2 and collagenase release from synoviocytes. Furthermore, synoviocyte proliferation was increased in the presence of the neuropeptide. Similar effects were observed with a truncated form of substance P. Synoviocytes were sensitive to very small doses of the neuropeptide ($10^{-9}M$), and its effects were inhibited by a specific antagonist. Thus, the specific stimulation of synoviocytes by the neuropeptide substance P represents a pathway by which the nervous system might be directly involved in the pathogenesis of rheumatoid arthritis.

MULTIPLE FACTORS APPEAR TO contribute to the pathogenesis of rheumatoid arthritis (RA), including genetic predisposition, impaired immunological control of virus infection, and autoantibody formation (*1*). In the synovium, activated lymphocytes and monocytes interact to generate interleukin-1 (IL-1). This lymphokine and monocyte-derived tumor necrosis factor–alpha (TNF-α) stimulate release of collagenase and prostaglandin E_2 (PGE_2) from synoviocytes (*2*).

Certain clinical findings are consistent with nervous system involvement in RA: (i) the distribution of synovitis is usually symmetric (*3*); (ii) joints in the paretic side of hemiplegic patients who later develop RA are spared from the inflammatory process (*4*); and (iii) onset and exacerbation of the disease are often preceded by psychological trauma (*5*). The basis for such effects in RA is unknown. Joints are innervated by corpuscular mechanoreceptors and plexus, as well as by free endings of small unmyelinated sensory afferent nerve fibers (*6*). These sensory fibers contain the neuropeptide substance P, which is involved in transmission of pain signals (*7*). Substance P is released in the spinal tract upon orthodromic stimulation, but is also released antidromically from the peripheral nerve terminals (*8*). This neuropeptide has multiple proinflammatory properties (*9*) and has been shown to increase the severity of adjuvant-induced arthritis in rats (*10*). The relative susceptibilities of knee and ankle joints to adjuvant arthritis are correlated with the densities of their sensory afferent nerve fibers (*10*). The nonneural target cell for the effects of substance P in joints is unknown, and the role of the peptide in RA has not been explored. We now report that in RA synoviocytes are stimulated by substance P.

The effect of substance P on the function of synoviocytes in RA was tested by measuring PGE_2 and collagenase production (*11*). Synoviocytes were isolated by collagenase-deoxyribonuclease treatment of synovial tissues obtained from RA patients undergoing joint replacement and were passaged once in culture before use in the experiments. We quantified by radioimmunoassay the levels of PGE_2 secreted during 48 hours of culture. Substance P, truncated substance P containing amino acids 4 to 11, and the antagonist [D-Pro^2, D-Phe^7, D-Trp^9]substance P (D–substance P) were added at start of culture.

Synoviocytes spontaneously released PGE_2 at 92 ± 14 ng/ml during 48 hours of culture (Fig. 1A). The addition of substance P increased PGE_2 release in a dose-dependent manner, with maximal stimulation occurring at $10^{-8}M$. A similar dose-response curve was obtained with substance P frag-

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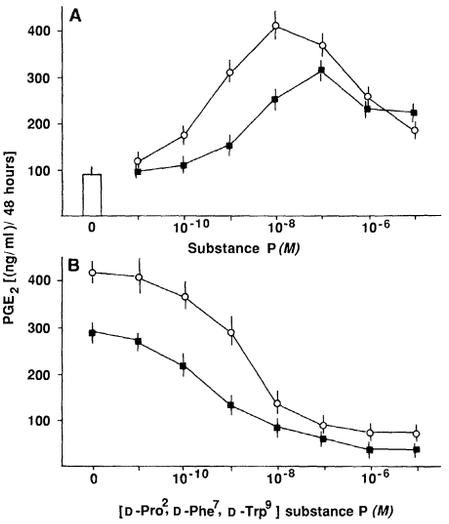

Fig. 1. Prostaglandin E_2 release from rheumatoid arthritis synoviocytes. In their second passage in culture, synoviocytes were dislodged by trypsin-EDTA from tissue culture flasks. Cells were then cultured at 2×10^4 per milliliter in 24-well cluster plates. The culture medium contained RPMI-1640, 2% heat-inactivated (56°C, 30 minutes) fetal bovine serum, penicillin (100 unit/ml), streptomycin (100 μg/ml), 2 m*M* L-glutamine, and polymixin B (12.5 μg/ml). (**A**) Synoviocytes were stimulated with substance P (○) and fragment 4–11 of substance P (■) (Peninsula Laboratories, Belmont, California). (**B**) The antagonist D–substance P was titrated against substance P at $10^{-8}M$ (○) and $10^{-9}M$ (■). Cells were cultured for 48 hours in an atmosphere of 5% CO_2– 95% air. Culture supernatants were collected, filtered through 0.45-μm filters, and stored at −70°C. Prostaglandins in the culture supernatants were extracted by adding HCl to reduce *p*H to 3.5. Three volumes of ethyl acetate were mixed with the supernatant, and the organic and aqueous phases were separated by centrifugation. The organic phase was removed and the ethyl acetate was evaporated. The residue was dissolved in buffer containing 0.01*M* phosphate and 0.1% bovine gamma globulin. PGE_2 was then quantified by radioimmunoassay (Seragen, Boston, Massachusetts). A standard curve was established for each assay with serial dilutions of PGE_2 between 2000 and 8.2 pg per 0.1 ml. Data from five separate experiments performed in triplicate are shown as PGE_2 in nanograms per milliliter, with bars indicating SEM.

ment 4–11, but its maximal effect was less than that of the full-length peptide.

D–Substance P has been shown to antagonize specifically the effects of substance P (*12*). To test for specificity of the substance P stimulation of synoviocytes, we titrated D–substance P against substance P at 10^{-8} and $10^{-9}M$. At both concentrations, the antagonist reduced the levels of PGE_2 (Fig. 1B). These results indicate that stimulation of synoviocytes by substance P is a specific function of this peptide.

Collagenase release from RA synoviocytes is an important pathogenetic process that leads to connective tissue destruction. To evaluate the effects of substance P on collagenase release, we collected synoviocyte culture supernatants and activated latent collagenase by short trypsin treatment (*11*). These activated supernatants were then exposed to a film of ^{14}C-labeled collagen (*13*). Collagen degradation was measured as ^{14}C released into the fluid phase in the collagen-coated plates. Synoviocytes spontaneously released collagenase activity, but this was augmented five- to eightfold with $10^{-8}M$ substance P. The effect of substance P was specifically antagonized by D–substance P (Table 1). Furthermore, D–substance P did not have a direct agonist effect. Interleukin-1 induces collagenase release from synoviocytes (*2*). We used recombinant human IL-1β (*14*) as a positive control and also as a test for specificity of the antagonist. D–Substance P did not reduce the IL-1–induced release of collagenase. Thus, D–substance P specifically competes with substance P, and the results suggest that the stimulation of synoviocytes by substance P and IL-1 occurs through distinct receptors.

Next, we evaluated whether the stimulation of collagenase release by substance P is selective or part of an overall increase of total protein synthesis. For this purpose, synoviocytes were stimulated with substance P and treated with a mixture of ^3H-labeled amino acids. Supernatants were collected after 48 hours, proteins were precipitated, and ^3H activity in the precipitate was measured. Total protein synthesis was stimulated by substance P, with maximal 5.5-fold augmentation occurring at $10^{-8}M$. This effect of substance P on protein synthesis was also specifically reduced by the antagonist. Since the levels of collagenase were increased by a similar factor (5.3), it appears that substance P increases total protein synthesis, including collagenase synthesis.

Finally, we examined the effects of substance P on proliferation of synoviocytes. Cells were cultured for 66 hours in the presence of substance P and then treated with bromodeoxyuridine (BrdUrd) for 6 hours. Cells were then trypsinized and stained with antibody to BrdUrd. Unstimulated synoviocytes contained 8.3 ± 2.3% of BrdUrd-positive cells whereas, in cells stimulated with substance P at $10^{-8}M$, the number increased to 29.6 ± 3.8%. D–Substance P antagonized the effect of substance P on proliferation (Table 2). When we used [^3H]thymidine incorporation as an alternative method to quantify synoviocyte proliferation, we observed similar effects.

Our results demonstrate that substance P is a potent and specific stimulus for multiple functions of RA synoviocytes in vitro. This is also likely to occur in vivo, since substance P can be released into tissues from peripheral nerve endings of the primary sensory neurons and substance P–immunoreactivity has been detected in synovial fluids (*15*).

Since the inflammatory process in RA is associated with the continued generation of mediators that activate the nerve endings of the nociceptive afferent nerve fibers, these fibers will contain increased concentrations of substance P (*16*) and may continually

Table 1. Collagenase release from rheumatoid arthritis synoviocytes cultured in medium alone (control) or in the presence of recombinant human IL-1β (100 ng/ml) or substance P ($10^{-8}M$). D–Substance P was added to the cultures at the indicated concentrations. Collagenase was assayed by the method of Johnson-Wint (*13*) as digestion of a ^{14}C-labeled collagen film. Type III collagen from calf skin (Sigma, St. Louis, Missouri) was labeled with [^{14}C]acetic anhydride (10 mCi/mmol). Fifty microliters of a [^{14}C]collagen solution (2 mg/ml) was added to each well in 96-well, flat-bottom tissue culture plates at a 45° angle. The plates were incubated for 1 hour at 37°C to polymerize the collagen and were then immersed in distilled water. The plates were then left overnight at room temperature to permit drying of the collagen films. Supernatants from synoviocytes that had been cultured for 48 hours were treated with trypsin (100 μg/ml) for 10 minutes at 25°C to activate latent collagenase. Soybean trypsin inhibitor was added to attain a final concentration of 500 μg/ml and the solution was left for 20 minutes at room temperature. Two hundred–microliter portions of solution were added in triplicate to the [^{14}C]collagen-coated wells. The plates were incubated at 37°C for 3 hours; 150 ml of the solution was then carefully removed and ^{14}C activity was counted by scintillation spectrophotometry. Each assay included a control consisting of fresh tissue culture medium that had been treated with trypsin and soybean trypsin inhibitor. The amount of radioactivity detected in these solutions after a 3-hour incubation on [^{14}C]collagen films was subtracted from the values obtained for the synoviocyte supernatants. Data from five experiments performed in triplicate are expressed as counts per minute ± SEM of degraded collagen.

D–Substance P (*M*)	Collagen degradation (count/min) in presence of		
	Control	IL-1β	Substance P
0	876 ± 52	6230 ± 496	4862 ± 354
10^{-5}	693 ± 48	7042 ± 634	628 ± 79
10^{-6}	756 ± 83	6529 ± 593	1538 ± 126
10^{-7}	906 ± 78	7282 ± 604	1842 ± 202
10^{-8}	852 ± 91	6832 ± 714	2984 ± 522
10^{-9}	792 ± 69	5986 ± 633	4043 ± 472

Table 2. Effect of substance P and D–substance P on synoviocyte proliferation. Synoviocytes were cultured in the presence of substance P and D–substance P ($10^{-8}M$) for 72 hours. For the last 6 hours, 5-bromo-2′-deoxyuridine (10 μM) and thymidine (32 μM) were added to the cultures. Cells were then dislodged with trypsin. After being washed with cold phosphate-buffered saline, the cells were fixed with 70% ethanol at 0°C for 30 minutes and spun onto microscope slides. DNA was denatured with 0.07*M* NaOH at room temperature for 2 minutes. Slides were then incubated with monoclonal antibody to BrdUrd (1:500). Bound antibodies were visualized with fluorescein isothiocyanate–labeled antibody to mouse immoglobulin. The cells were analyzed with a Leitz OrthoLux II microscope. Results from four experiments performed in triplicate are expressed as the percentage of fluorescence positive cells ± SEM.

Substance P (*M*)	Cells stained with antibody to BrdUrd	
	Alone	In presence of D–substance P
0	8.3 ± 2.3	
10^{-6}	18.3 ± 3.4	
10^{-7}	24.8 ± 3.9	
10^{-8}	29.6 ± 3.8	13.6 ± 2.8
10^{-9}	19.8 ± 3.2	
10^{-10}	14.4 ± 2.9	

release substance P into the joint tissues. From the present results it appears that long-term exposure of synoviocytes to substance P could contribute to the development of several pathological findings in RA. By stimulating synoviocyte proliferation, substance P can enhance the formation of pannus, which represents excessive growth of synovial tissue over the joint surface. This hypothesis is consistent with its known stimulation of skin fibroblast proliferation (*17*). The basis for the effects of substance P on these cells may be the amino acid sequence homology between substance P and acidic fibroblast growth factor (*18*).

The augmentation of collagenase release and PGE_2 generation from synoviocytes activated by substance P suggests additional roles for this neuropeptide in RA. Through release of collagenase, substance P may stimulate loss of cartilage and development of lesions in the adjacent bone. Through PGE_2 release, substance P may provide a signal to perpetuate the inflammatory process in the arthritic joint. The two other known activators of synoviocytes, IL-1 and TNF-α, are both products of the reticuloendothelial system. An interaction of the nervous and reticuloendothelial system involving substance P has been proposed (*9*).

In summary, these observations define a specific pathway by which substance P, a nervous system–derived peptide, can contribute to the pathogenesis of RA. Similar effects of substance P on connective tissue remodeling and destruction may be relevant also in the response of other organs to inflammatory or traumatic injury, as well as inflammation in other diseases.

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Recombinant Interferon Enhances Monoclonal Antibody–Targeting of Carcinoma Lesions in Vivo

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Heterogeneity in the expression of tumor-associated antigens, as defined by the binding of monoclonal antibodies, is a characteristic common to most, if not all, human carcinoma cell populations. Antigen-negative cells within the population can escape detection and therapy by their failure to bind the appropriate antibody. Therefore, the extent of antigenic heterogeneity is an important consideration when designing protocols for the management of cancer by administration of monoclonal antibodies. One approach to counteracting the effect of antigenic heterogeneity is the use of clone A of recombinant human leukocyte interferon (Hu-IFN-αA). Administration of Hu-IFN-αA in vivo effectively increased the amount of tumor antigen expressed by a human colon xenograft in situ and augmented the localization of a radiolabeled monoclonal antibody to the tumor site. Concomitant administration of Hu-IFN-αA and monoclonal antibody may thus be effective in overcoming the antigenic heterogeneity of carcinoma cell populations and in enhancing the efficacy of monoclonal antibodies in the detection and treatment of carcinoma lesions.

HETEROGENEITY OF ANTIGEN EXpression is characteristic of human carcinoma cell populations (*1*). This phenomenon has far-reaching implications for the use of monoclonal antibodies (mAbs) in diagnosis or therapy. Rendering a human tumor cell population more homogeneous for the expression of an antigen could result in an augmentation of mAb binding and, thus, more efficient diagnostic or therapeutic results.

Human natural and recombinant interferons, in addition to eliciting antiviral and antiproliferative actions, can alter the surface antigenic phenotype of various human target cells in vitro (*2–6*). The level of expression of class I and II histocompatibility antigens as well as of certain tumor-associated antigens can be modulated by recombinant human interferons (*2, 4–6*). Recombinant human leukocyte interferon (Hu-IFN-α) can enhance the expression of histocompatibility antigens (HLA) and of several mAb-defined tumor antigens on the surface of human breast and colon carcinoma cells (*5, 7*). We reported recently that of eight different recombinant species of Hu-IFN-α,

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