Table 3. ^{3}H diazepam binding of imprinted and not imprinted *Tetrahymena* pellicle related to the control as 100

Binding %		Binding %	
Control	100	100	Imprinted
Control + 1000 × cold diazepam	100	59.0*	Imprinted + 1000 × cold diazepam

* = $p < 0.05$

fact taken place in *Tetrahymena*. It deserves special mention that the difference between the cells imprinted and not imprinted with diazepam was still demonstrable after one week, in which about 50 generation changes occurred. Thus imprinting maintained a 'memory' of primary interaction with diazepam over many progeny generations, to judge from the greater binding and uptake of the drug by these relative to the control.

The plasma membrane preparations represented an entirely different experimental system, in which incorporation of ^{3}H-diazepam into the cells was a priori impossible. Displacement of the ^{3}H-diazepam molecules bound to the membrane of the not-pretreated cells failed to take place in that system, whereas more than 40% ($p < 0.05$) of bound ^{3}H-diazepam was displaced on the imprinted cells in presence of an 1000-fold amount of unlabeled (cold) diazepam (table 3). Although these observations fail to substantiate the specificity of the spontaneous binding sites, such as are presented by the control cells, they substantiate unequivocally the establishment of diazepam imprinting in *Tetrahymena*, and the specificity of the receptors induced by imprinting.

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Inhibition of collagen synthesis by interleukin-1 in three-dimensional collagen lattice cultures of fibroblasts

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Summary. Interleukin-1 (Il-1) was added to collagen lattice cultures of human skin fibroblasts. No cell division was induced, the ability of fibroblasts to contract the lattices was decreased and a dose-related inhibition of collagen synthesis without effect on non-collagen proteins was found. Indomethacin had no influence on these effects.
Key words. Collagen lattice culture; collagen synthesis; interleukin-1; indomethacin; fibroblast culture.

Cultures of fibroblasts in collagen lattices, which have been described by several authors [1–3], constitute a valuable tool for the study of the behavior of cells embedded in an extracellular matrix, in a physiological situation comparable to the one which they occupy in vivo. In this complex medium, it has been demonstrated that fibroblasts remain more or less quiescent. They do not divide, and their rate of collagen synthesis is reduced to a negligible level compared to their activity in monolayer cultures [4]. A very noticeable property of these lattice cultures is the contraction which operates during the first days of culture and results in the structuring of a resistant, organized, coherent lattice [5, 6]. The mechanism of this lattice contraction is not well understood at the present time. On the other hand, many efforts have been devoted to establish the reasons why cells are in apparent dormancy in these collagen lattices. For instance, several growth factors have been tried, more or less successfully, in order to restart cell division and protein synthesis in lattice cultures [7, 8]. Up to now, interleukin-1 (Il-1), a cytokine capable of modulating a number of biological activities of fibroblasts, has not been used in that system. Il-1 is shown to be a multifunctional factor, acting as a lymphocyte activator, an inducer of acute phase protein production and an endogenous pyrogen [9]. In connective tissue, Il-1 induces the secretion of proteolytic enzymes including collagenase [10] plasminogen activator [11] and stromelysin [12], a process that promotes matrix breakdown. Il-1 also stimulates the production of prostaglandin E_2 by mesenchymal cells [10]. On the other hand, Il-1 is a well-known mitogenic factor for fibroblastic cells [13]. Furthermore, Il-1 has been reported to increase the steady-state level of collagen mRNAs in fibroblasts [14, 15], synovial cells [14, 16] and chondrocytes [14]. However, contradictory results have been obtained in studies on the effect of Il-1 on collagen protein production [14–21]. This discrepancy can be explained by variations in the experimental procedures and the cell lines used [22]. In particular, it has been suggested that the Il-1 induced prostaglandin PGE_2 release could be responsible for an inhibition of collagen synthesis,

at least in cell types which produce high amounts of that prostaglandin[14,16]. In support of this explanation, it is known that PGE_2 activates the intracellular formation of cyclic AMP which usually inhibits collagen synthesis and promotes its intracellular degradation[23]. Moreover, exogenous PGE_2 has recently been shown to decrease collagen mRNA level in cultures of dermal fibroblasts[24].

Taking into account that experimental conditions could influence the response of connective tissue cells to Il-1, it was of interest to study fibroblasts seeded in lattice cultures to investigate their division, protein synthesis and effect on lattice contraction and to consider, in addition, the effect of treating these cultures with indomethacin, in order to suppress the formation of PGE_2 triggered by Il-1. We present data showing that Il-1 β had inhibiting effects on both lattice contraction and fibroblast collagen synthesis. Addition of 10 µM indomethacin was without effect on these functions.

Materials and methods. Chemicals were purchased from Prolabo (Paris, France), unless another source is mentioned. Ascorbic acid was bought from Merck (Darmstadt, FRG), indomethacin and β-aminopropionitrile (fumarate) from Sigma (St Louis, USA), L-proline and L-glutamine were obtained from Calbiochem (Meudon, France) and L-[U-^{14}C]-proline (specific activity over 9.25 GBq/mmol) from New England Nuclear (Paris, France). Triton X-100 was bought from Technicon (Tarrytown, USA). All reagents for cell culture were purchased from Gibco (Paisley, Scotland) and plastic petri dishes from Flow (Irvine, Scotland).

Bacterial collagenase (CLSPA grade) was obtained from Worthington (Freehold, USA) and purified in the laboratory according to Peterkofsky and Diegelmann[25].

Interleukin-1 from human monocytes, purified according to Rosenwasser et al.[26] was supplied by Genzyme (Boston, USA). This preparation was free from lipopolysaccharide, interleukin-2 and interferons. One unit is defined as the amount of Il-1 required to double the proliferative response of mouse thymocytes stimulated with 1 µg/ml phytohemagglutinin. No indication is given by the supplier on the relationship between units and weight for this preparation, so that concentrations of Il-1 were expressed only in terms of units/ml. Preliminary studies with this preparation have shown that the range of efficiency on monolayer fibroblast cultures was 0.01–5 U/ml[20], which should correspond to 0.1–50 µg/ml[15]. This preparation of Il-1 is supposed to contain essentially Il-1 β since blood monocytes have been shown to produce about 9 times more Il-1 β than Il-1 α.

Cell cultures. Fibroblast strains were established in our laboratory from explants of human foreskin, obtained with parental consent. They were grown by using routine techniques[27] in Eagle's essential medium (MEM) supplemented with 10% fetal calf serum (FCS). For seeding into collagen lattices, cells from subcultures 3 to 10 were used.

Prior to the seeding into lattices, the confluent fibroblasts were detached from their culture flasks by trypsinization, collected by centrifugation for 10 min at 800 × g, and diluted to a convenient cell density with Dulbecco's modified Eagle's medium (DMEM).

Collagen lattices were prepared according to a previously described technique[5,28], slightly modified as follows (all the components being sterilized before use): 1.25 ml of stock incubation medium (containing 0.7 ml of 2.5 × DMEM, 0.2 ml of FCS, 0.2 ml of twice-distilled water and 0.15 ml of 0.06 M NaOH) at 37 °C was poured into a 35-mm-diameter plastic petri dish. The pepsin-digested calf skin collagen[29] (made sterile by precipitation in 70% ethanol) was dissolved at 2 mg/ml in 0.018 M acetic acid solution and kept at 4 °C before use. 0.5 ml of the solution was added simultaneously with the fibroblast suspension (0.25 ml) on seeding. After gentle stirring, lattices formed in less than 5 min at 37 °C.

Lattice contraction. The lattice diameter was measured every day, with the dish placed on a black background. The contraction of the lattice was evaluated by: (a) half-contraction time: time necessary for cells to contract the lattice down to the half of their initial area, expressed in hours. (b) 5 day-area: area of lattice at day 5 after seeding, expressed in mm^2.

Cell counting. After various periods of incubation (for duration, see results), the cells were recovered from collagen lattices by digestion of the network with 10 U/ml purified bacterial collagenase (2 h at 37 °C) and counted with a Malassez cell.

Protein and collagen syntheses. Protein synthesis were studied by ^{14}C-proline incorporation during 5 days of culture. For that purpose, the incubation medium was supplemented on seeding with 50 µg/ml β-aminopropionitrile, 50 µg/ml ascorbic acid, 74×10^3 Bq/ml of ^{14}C-proline and 0.2 mM unlabelled proline.

After incubation, the supernatant was obtained by centrifugation at 10,000 × g and the lattice rinsed with DMEM. The supernatant and rinses were pooled and constituted the S fraction. Cells and lattices were dissolved together by treatment with a 0.2% Triton X 100 solution for 1 h at 65 °C and heated 10 min at 100 °C in order to inhibit lysosomal enzymes. This suspension represented the fraction C + L.

Aliquots of the fractions S and C + L were counted for total radioactivity in a Minaxi type 4430 Packard scintillation counter. The remaining solutions were extensively dialyzed against bidistilled water and precipitated by addition of ammonium sulfate to 1.33 M. The precipitates were suspended in 1 ml of 0.1 M ammonium formate buffer pH 7.8, containing 5 mM calcium acetate and bacterial collagenase (20 µg/ml in S fraction, 50 µg/ml in C + L fraction). This mixture was incubated for 18 h at 37 °C and the non-collagenous proteins were precipitated by addition of ethanol to give a final concentration of 90% (v/v). The radioactivity incorporated in the collagen fraction (supernatant) was then evaluated by scintillation counting.

Statistical calculations. All the experiments were performed in quadruplicate. The statistical significance was calculated by the Student's t-test.

Results. As shown in table 1, Il-1 did not cause any significant variation in cell division, when used at concentrations 1.25, 2.5 and 5.0 U/ml. The number of fibroblasts measured in control lattices after 5 days of culture was not different from the number of seeded cells (+8.6%, not significant). None of the Il-1 concentrations tested modified this number.

The effect of Il-1 on the lattice contraction was studied in cultures seeded with 10^5 fibroblasts, in the presence of 5% fetal calf serum, during 5 days of incubation. The curves of contraction are shown in figure 1. The initial rate of contraction was not modified by Il-1 (1.25 to 5.0 U/ml): the calculated half-contraction times were not different in the 4 series (table 2). By contrast, Il-1 caused a significant inhibition of

Table 1. Effect of Il-1 on fibroblast number. Fibroblasts were counted 5 days after seeding. Incubation in the presence of 5% fetal calf serum; addition of Il-1 simultaneously with seeding. Results represent the mean of quadruplicate determinations ±1 SEM. No significant differences.

	Il-1 concentration (U/ml)			
	0	1.25	2.5	5.0
Number of seeded cells	101 000 ± 2500	101 000 ± 2500	101 000 ± 2500	101 000 ± 2500
Number of cells on day 5 after seeding	109 690 ± 4430	108 280 ± 6730	102 190 ± 8090	103 750 ± 6030

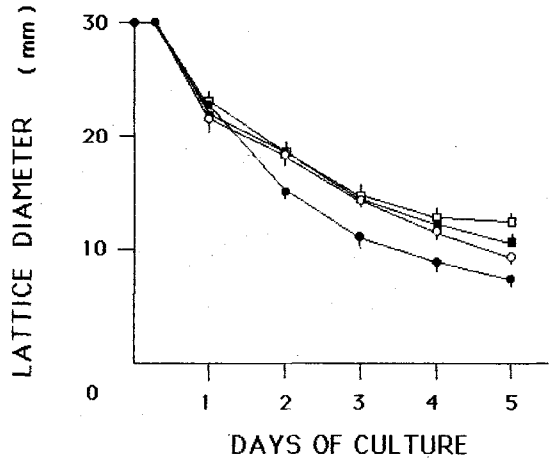

Figure 1. Effect of Il-1 concentration on the contraction of collagen lattices by human fibroblasts in the presence of 5% FCS and 0 (●, control), 1.25 (○), 2.5 (■) and 5.0 (□) U/ml Il-1. Each point represents the mean of quadruplicate determinations ±1 SEM.

the contraction after the 1st day of incubation. This effect was earlier and more intense with increasing concentrations of Il-1. The area of the lattices after 5 days of contraction remained broader in all the series performed in the presence of Il-1 at concentrations from 1.25 to 5 U/ml (table 2).

We checked the influence of the concentration of Il-1 on protein synthesis by fibroblasts cultured in collagen lattices. No variation of total protein synthesis was found for Il-1 concentrations ranging from 1.25 to 5 U/ml (fig. 2A). By contrast, Il-1 significantly inhibited collagen synthesis in the 3 series; these inhibitions were respectively −33%, −43% and −48% in comparison with controls (fig. 2B).

An experiment was performed in the presence of 10 μM indomethacin (fig. 3), an inhibitor of the cyclooxygenase pathway of arachidonate metabolism. Indomethacin alone caused no modification of total protein and collagen syntheses. It did not reverse the inhibitory effect of Il-1 on collagen synthesis, when added simultaneously.

Discussion. In recent years it has become evident that, besides its effects on immune cells, Il-1 is able to modulate the synthesis and breakdown of the extracellular matrix through different biological activities affecting the mesenchymal cells. As a consequence, it has been suggested that it plays a pivotal role in several pathological situations, including inflammation.

As regards collagen production, the effect of Il-1 has been studied by several laboratories on monolayer cultures of fibroblasts, with variable results. Today, we have clear evidence that Il-1 increases the level of collagen mRNAs [14, 15], but this elevation is not always associated with a parallel increase in the protein secreted in the medium [16]. This seems to be dependent on the experimental conditions used, such as the presence or absence of serum in the culture medium. Thus, some doubt remains about the exact role of Il-1 in vivo on collagen production. Therefore, it is of interest to study the effect of Il-1 on fibroblasts cultured in collagen lattices,

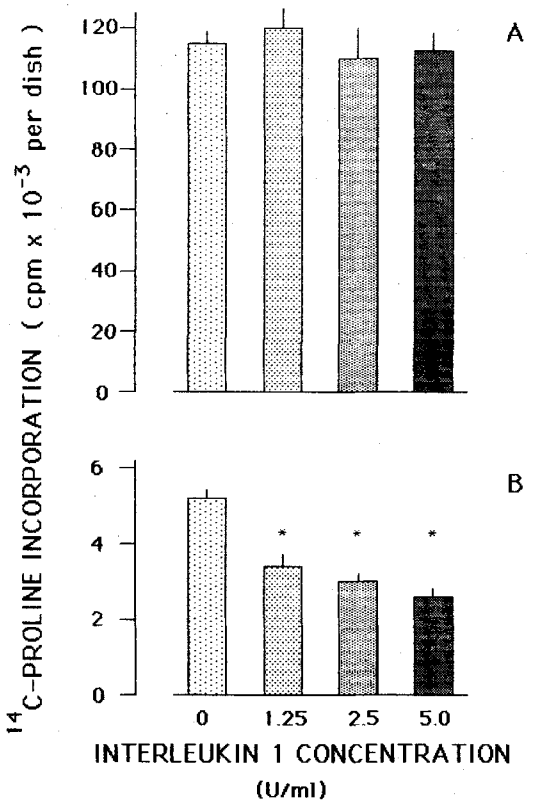

Figure 2. Effect of Il-1 concentration on total protein (*A*) and collagen (*B*) syntheses by 100,000 fibroblasts cultured in collagen lattices during 5 days in presence of 5% FCS. The data presented show the means of quadruplicate determinations ±1 SEM.* Significantly different from controls ($p < 0.001$).

because these culture conditions are more germane to the in vivo situation of fibroblasts.

In conventional monolayer fibroblast cultures, Il-1 has been shown [14, 15, 20, 21] to exert its effect on collagen synthesis in the concentration range of 0.1–10 U/ml. Therefore we decided to use similar doses in our experiments. We found that human foreskin fibroblasts submitted to these various concentrations of a preparation of monocyte Il-1 exhibited a significant decrease in their ability to contract collagen lattices. In addition, the synthesis of collagen was significantly inhibited in a dose-dependent fashion, whereas that of non-collagen protein was not modified. In order to determine whether this inhibition was prostaglandin-dependent, we studied the effect of Il-1 in the presence of indomethacin, which blocks prostaglandin synthesis. The inhibition of collagen secretion was of the same magnitude in the presence as in the absence of the drug, suggesting that prostaglandin E_2 is not involved in the mechanism of Il-1 action on collagen synthesis.

It is worth noting that the present data obtained in lattice cultures are in good agreement with a recent report on the in vivo effect of Il-1 on experimental granuloma [30]. In this

Table 2. Effect of Il-1 on collagen lattice contraction. The lattices were seeded with 100 000 fibroblasts, in the presence of 5% fetal calf serum and the mentioned concentrations of Il-1. The results represent the mean of quadruplicate determinations ±1 SEM. Result significantly different from control: **$p < 0.01$; ***$p < 0.001$.

	Il-1 concentration (U/ml)			
	0	1.25	2.5	5.0
Half-contraction time (h)	29.5 ± 2.5	29.9 ± 2.5	31.8 ± 4.9	31.4 ± 2.1
5-d area (mm^2)	41.4 ± 2.9	71.4 ± 7.9**	82.6 ± 4.1***	109.9 ± 2.6***

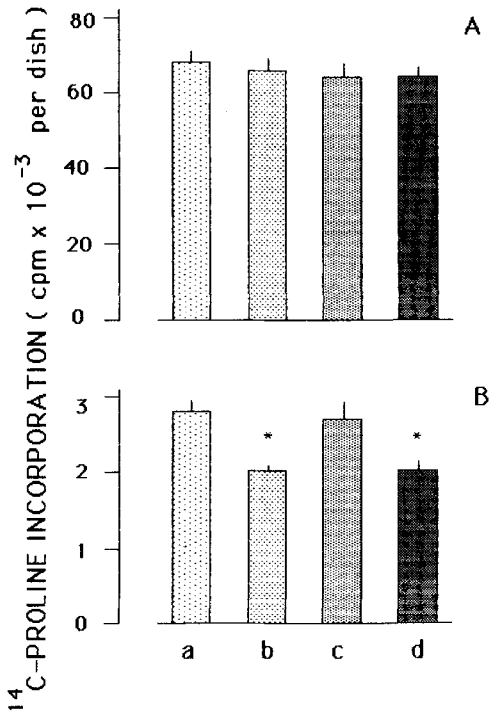

Figure 3. Absence of effect of indomethacin on protein syntheses and Il-1-induced inhibition of collagen synthesis. The columns represent the syntheses of total protein (*A*) and collagen (*B*) by 100,000 fibroblasts cultured during five days in collagen lattices in the presence of 5% FCS. a: control, b: Il-1 1.25 U/ml, c: indomethacin 10 μM, d: Il-1 1.25 U/ml + indomethacin 10 μM. *significantly different from controls ($p < 0.001$).

work, Il-1 was injected for seven days into viscose cellulose sponges implanted under the dorsal skin of rats. Il-1 significantly decreased the hydroxyproline content of granulomas, compared to controls receiving saline solution. Moreover, the cells obtained from the granulation tissue and cultured in vitro responded in the same way to Il-1, their collagen production being reduced to 80% of that in controls. It must be noticed also that the authors did not observe any effect of Il-1 on cell proliferation in their cultures, as we did in our system.

The inhibition of lattice contraction induced by Il-1 may be ascribed to the phenomena of decreased adhesion and mobility of the fibroblasts, as it seems at present that the contraction of lattices depends on the action of the cells and more particularly of their cytoskeleton [1, 31, 32]. It is also likely that the response of embedded fibroblasts to Il-1 is somehow related to their quiescent situation. It has been previously shown that Il-1 did not stimulate DNA synthesis in quiescent fibroblasts in monolayer cultures [33].

The decrease of collagen synthesis has to be related to the phenomena of the first stage of inflammation, when most of the components of connective tissue in the inflamed area are submitted to fast degradation. During this catabolic step, most if not all syntheses are found to be inhibited. The two associated phenomena of inhibition of lattice contraction and decrease of collagen synthesis depend probably on some kind of resting period induced by Il-1 at the beginning of inflammation. It is likely that the effect of Il-1 is reversed or abolished when inflammation progresses to the following steps of reconstitution of the tissue, characterized by the layering of newly synthesized collagen fibers. Furthermore, we cannot exclude that Il-1 could enhance collagen synthesis later in the process since it is becoming clear that one factor can have both stimulatory and inhibitory activity, depending on the context provided by the other signal molecules present in the target cells.

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