Connective Tissue Activation

IV. Regulatory Effects of Antirheumatic Drugs

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Cortisol, phenylbutazone, indomethacin, acetylsalicylic acid, meclofenamic acid, flufenamic acid and mefanamic acid were potent inhibitors of connective tissue activation, especially if introduced into synovial cultures within 2 hours of initiation of the activation process. Colchicine, chloroquine and gold thiomalate were ineffective, while hydroxychloroquine and sodium salicylate exhibited modest inhibitory capacity. Most of the active agents exhibited suppressive capacity at concentrations similar to those used clinically. The suppressive capacity of the various drugs was reduced 20 to 60% by the presence of serum. Antirheumatic drugs do not appear to alter the activity of the stimulatory connective tissue activating peptide molecule itself, but may act by interfering with the transcriptional and translational components of the connective tissue activation sequence.

Development of rational therapy to interdict self-perpetuating, destructive inflammatory processes will require precise knowledge of the interlocking components of these phenomena. The clotting process, kinin generation, activation of the complement sequence, release of lysosomal enzymes and phagocytic activity are interwoven in a complex and poorly understood manner. Most of these component processes are incompletely understood in themselves, and their reacting constituents are only partially characterized in chemical terms. Little critical information exists concerning the factors regulating the interaction of these interlocking systems, and even the relative importance of the components listed is not clear. Conventional views of inflammation suggest that acute (exudative) phenomena primarily embrace altered behavior of the microcirculatory system, including the vascular endothelium itself, leukocytes, platelets and soluble plasma constituents. Reparative phenomena derive from the activity of connective tissue cells and occur later in the inflammatory process when formation of glycosaminoglycans and collagen is detected. This laboratory reported a polypeptide which may function to initiate the transition from the exudative to the reparative phase of the inflammatory process (1). This activator polypeptide (CTAP) is derivable from exudative cells (2, 3) and induces hypermetabolic activity in synovial cells, including markedly increased glycolysis and accelerated formation of hyaluronic acid (1, 3), a process we have termed "connective tissue activation." Recently, it has

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been shown that the peak of CTAP activity in cotton pellet granulomata occurs early, when the transition from the acute to the reparative stage of this inflammatory reaction is seen microscopically (4). While chronic, self-perpetuating inflammation clearly involves continuation of both the exudative and reparative phases of the process, the latter, with its associated elaboration of collagenase, may be a dominant factor in the tissue destruction seen with protracted inflammation (5). It would therefore be of interest to determine whether antirheumatic drugs interfere with connective tissue activation, a possibly essential step in the connective tissue contribution to the inflammatory process. Preliminary work reported by this (6–8) and other (9) laboratories suggests that cortisol and other antirheumatic drugs may modify connective tissue activation in vitro. This report deals in a more comprehensive manner with a wider range of drugs and approaches certain aspects of their mode of action.

MATERIALS AND METHODS

Tissue Culture Methods. Culture methods have been described in detail earlier (10–12). Synovial tissue was obtained at amputation or arthrotomy and dissected into 1 × 2 mm explants which were immobilized under perforated cellophane in T-9 or T-15 flasks. The synovial cell strains developed for this study came from normal joints and patients with degenerative joint disease or traumatic synovitis. Routine culture medium consisted of medium 1066 (80%), fetal calf serum (10%) and heat-inactivated human serum (10%) supplemented with 100 μg each of penicillin G and streptomycin sulfate, as well as 0.002 mM L-glutamine. Nutrient medium was changed completely every 48 to 72 hours. When sufficient outgrowth of fibroblastic cells had occurred, it was dispersed with 0.1% trypsin and used to initiate monolayer cultures in serum dilution bottles. Cell dispersion was accomplished with crystalline trypsin,* 1.0 mg/ml, and enumeration was carried out with a Coulter Model B electronic cell counter. Aliquots of explant material and dispersed cells were stored by freezing with 6% dimethylsulfoxide (DMSO), as noted earlier (12). To avoid the unpredictable behavior of senescent cell strains, we restricted use of cell strains to the first ten trypsin subcultures following derivation from primary explants.

Chemical Measurements. Hyaluronic acid was measured in both serum-containing (13) and serum-free media (1) by methods involving isolation of the polymer followed by determination of the uronic acid moiety with a modified carbazole procedure (14). Glucose was measured by a glucose oxidase method (15) and lactate by the Barker-Summerson procedure (16). Protein was determined by the method of Oyama and Eagle (17).

Preparation of Connective Tissue Activating Peptide. Except where otherwise indicated, the CTAP used in these experiments was prepared from human spleen by homogenizing the tissue in 10 volumes of buffer containing 0.15 M sodium chloride, 0.05 M phosphate buffer, pH 7, and 0.1% β-mercaptoethanol. Following homogenization of splenic tissue in a Waring blender, the mixture was stirred for several hours at 4° C and gross particulate material removed by centrifugation at 5° C. The supernatant fraction was applied to a Sephadex G-50 column, and a broad zone of retained material was collected and the volume reduced by rotary evaporation at 30° C. This was dialyzed against buffered saline containing 0.0005 M cysteine and the volume adjusted so that 1.0 ml of extract represented 1.0 g of the original splenic tissue.

Antirheumatic Drugs. Antirheumatic agents included flufenamic acid (Lot No. X9063), mefenamic acid (X8726) and meclofenamic acid (X40590), supplied by Parke, Davis and Co. Indomethacin (L590266-0-70) and aurothiomalate (L413984-01A17) were provided by Merck Sharp & Dohme Research Laboratories. Phenylbutazone (SN15064) was a gift of the Geigy Corporation. Chloroquine diphosphate (N37458) and hydroxychloroquine sulfate (NO69RK) were obtained from the Winthrop Laboratories.

Experimental Protocols. Most of the experiments utilized the procedure previously outlined for the measurement of CTAP activity (1). In general, cultures were set out with 1 × 10^6 normal synovial fibroblasts per T-15 flask in a serum-containing medium. After a 4- to 6-hour interval to allow cell attachment to the glass, the serum-containing medium was removed and replaced by an assay medium consisting of Eagle's synthetic medium buffered with HEPES buffer.* A typical experiment would include 4 groups of flasks: Group 1 including control cells treated with a vehicle; Group 2, cells treated with test drugs; Group 3, cells treated with CTAP sufficient to induce a high level of activation; and Group 4, cells treated with CTAP and antirheumatic drugs whose efficacy as a blocker of "activation" was under test. Unless otherwise noted, the CTAP and drugs were added simultaneously.

*Worthington Biochemical Corporation, Freehold, New Jersey.

*N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid, from Calbiochem, Los Angeles, California.

Table 1. Failure of Colchicine and Chloroquine to Impede the Activation Process*

Assay cell strain	Additives	Glucose uptake (μM/mg cell protein/24 hr)	Lactate output (μM/mg cell protein/24 hr)	HA† synthesis rate (μg HA/mg cell protein/24 hr)	Incremental HA synthesis rate	Reduction of incremental HA (%)
HH-H	Vehicle	1.67 (1.44–1.95)	2.17 (1.09–3.26)	6.6 (4.6–8.5)	—	—
HH-H	CTAP	7.67 (7.52–7.97)	14.65 (14.11–15.46)	124.7 (120.4–129.1)	118.1	—
HH-H	Colchicine, 1.0 μg/ml	1.67 (1.44–1.78)	2.35 (1.63–3.53)	8.2 (6.7–10.6)	—	—
HH-H	Colchicine, 1.0 μg/ml + CTAP	7.46 (7.28–7.75)	11.39 (9.49–13.29)	119.9 (117.1–124.8)	111.7	5
HH-H	Chloroquine diphosphate, 2.0 μg/ml	1.61 (1.44–1.78)	3.71 (3.26–4.07)	7.2 (5.9–8.2)	—	—
HH-H	Chloroquine diphosphate, 2.0 μg/ml +CTAP	7.70 (7.51–7.91)	13.38 (12.48–14.11)	128.1 (120.2–132.2)	120.9	0

*The data are recorded as the mean of triplicate determinations, and the range is shown in parentheses. The assay medium did not include serum.

†HA = hyaluronic acid

Usually, culture groups were planted in triplicate; and the data appearing in the tables are the mean of the observations, while the ranges of the observations appear in parentheses. Activation of a connective tissue cell strain is reflected by a marked increase in the hyaluronic acid synthesis rate measured as incremental hyaluronic acid per mg cell of protein per 24 hours of incubation time. Drugs which effectively interfere with the activation process reduced the incremental hyaluronate synthesis induced by CTAP.

RESULTS

Colchicine and Chloroquine. Colchicine added to synovial cultures in a concentration of 1.0 μg/ml failed to impede the activation process stimulated by a simultaneously introduced CTAP preparation (Table 1). The concentration of colchicine employed was ample to produce metaphase arrest in cultivated synovial cells and is in excess of plasma concentrations achieved during therapy with this agent in humans. Not only was the burst of hyaluronate formation during the activation process unaffected by the presence of colchicine, but accelerated glucose uptake and lactate output were also unaffected. In a similar experiment, chloroquine diphosphate, 2.0 μg/ml, was shown to be without significant effect on the activation process (Table 1). The last column of Table 1 indicates that the added drugs did little to reduce the incremental hyaluronate synthesis incident to activation. The chloroquine diphosphate concentration, although greater than that achieved in human patients treated with this agent (18), produced no obvious morphologic alterations in the synovial cells which could be visualized by the light microscope in the living cultures. This latter observation contrasts with the situation with colchicine-treated cultures where the cells exhibited marked rounding.

Aurothiomalate and Phenylbutazone. The addition of aurothiomalate (1.0 to 6.0 μg/ml of culture medium) failed to depress the accelerated uptake of glucose, enhanced lactate

Table 2. Effect of Gold and Phenylbutazone on Connective Tissue Activation*

Assay cell strain	Additives	Glucose uptake (μM/mg cell protein/24 hr)	Lactate output (μM/mg cell protein/24 hr)	HA† synthesis rate (μg HA/mg cell protein/24 hr)	Incremental HA synthesis rate	Reduction of incremental HA (%)
HH-E	0	2.01 (1.87–2.14)	5.05 (4.28–6.03)	5.6 (4.2–7.9)	—	—
HH-E	CTAP	7.88 (7.82–7.92)	14.59 (12.24–16.33)	31.6 (24.9–35.2)	26.0	—
HH-E	Gold,‡ 4.0 μg/ml	2.56 (2.48–2.71)	4.27 (2.78–5.19)	6.1 (4.9–7.1)	—	—
HH-E	CTAP + Gold, 4.0 μg/ml	7.19 (7.12–7.24)	12.87 (12.39–13.82)	35.6 (31.4–39.6)	29.5	0
HH-E	CTAP + Gold, 6.0 μg/ml	6.57 (6.43–6.75)	12.09 (11.87–12.51)	32.0 (30.4–34.7)	25.9	0
HH-E	CTAP + Phenylbutazone, 75.0 μg/ml	5.15 (4.83–5.34)	7.45 (7.00–7.97)	10.5 (9.7–11.4)	4.9	81

*The assay medium did not include serum.
†HA = hyaluronic acid
‡Aurothiomalate

output and increased hyaluronate synthesis incident to addition of CTAP to test cultures (Table 2). These concentrations include and exceed those found in rheumatoid patients treated by chrysotherapy (19). On the other hand, it is clear from Table 2 that phenylbutazone substantially depresses all of the measured parameters of connective tissue cell activation at concentrations effective in clinical usage (20).

Salicylates, Hydroxychloroquine, Indomethacin and Cortisol. Many of the commonly used antirheumatic drugs were tested simultaneously in CTAP-activated synovial cell cultures with the results illustrated in Table 3. This experiment provided evidence for strong inhibition of the activation process by acetylsalicylic acid, indomethacin, phenylbutazone and cortisol. On the other hand, hydroxychloroquine and sodium salicylate appeared to be without suppressive effect. Salicylates (21), indomethacin (22) and phenylbutazone (20) were added in concentrations approximating those achieved in the plasma of patients undergoing treatment with these agents, while hydroxychloroquine (18) was used in a supratherapeutic concentration.

Effect of Serum on the Action of Antirheumatic Drugs. To assess the possibility that serum proteins might significantly alter the action of the antirheumatic drugs, parallel assays were performed in which one group of flasks was studied using the usual serum-free media, while a second group included 10% fetal calf serum and 10% human serum. It was clear that for the potent drugs such as indomethacin, phenylbutazone, acetylsalicylic acid and cortisol, serum does partially block the suppressive effects of the antirheumatic drugs. As noted in Figure 1, hydroxychloroquine and sodium salicylate have very little suppressive action regardless of whether serum is present.

Dose-Response Studies. The ability of cortisol in a serum-free assay system to suppress synovial cell activation was studied over a concentration range from 0.005 to 1.0 μg/ml. As shown in Figure 2, there was a significant suppression of synovial cell activation at the lowest

Table 3. Effect of Antirheumatic Drugs on Activation in Absence of Serum

Assay cell strain	Drug added (μg/ml)	CTAP added	Glucose uptake (μM/mg cell protein/24 hr)	Lactate output (μM/mg cell protein/24 hr)	HA synthesis rate (μg HA/mg cell protein/24 hr)	Reduction of incremental HA (%)
HH-I	0	0	2.39 (2.26–2.52)	3.30 (3.30–3.30)	9.1 (8.4–9.7)	—
HH-I	0	+	10.63 (10.16–11.09)	18.10 (16.70–19.50)	103.3 (100.3–106.3)	—
HH-I	Hydroxychloroquine, 2.0	0	2.95 (2.85–3.04)	4.76 (4.60–4.91)	8.4 (7.7–9.0)	—
HH-I	Hydroxychloroquine, 2.0	+	11.34 (11.25–11.43)	21.94 (21.91–21.97)	110.4 (105.4–115.4)	0
HH-I	Sodium Salicylate, 150	0	2.45 (2.29–2.61)	2.51 (2.34–2.67)	8.4 (8.2–8.6)	—
HH-I	Sodium Salicylate, 150	+	10.23 (9.26–11.19)	18.10 (17.03–19.17)	111.7 (108.4–114.9)	0
HH-I	Acetylsalicylic acid, 150	0	1.63 (1.33–1.92)	1.34 (1.15–1.52)	9.1 (8.3–9.9)	—
HH-I	Acetylsalicylic acid, 150	+	6.37 (5.15–7.58)	11.43 (10.18–12.68)	30.6 (27.2–34.0)	77
HH-I	Indomethacin, 15.0	0	2.62 (2.33–2.91)	3.52 (3.36–3.67)	10.3 (9.0–11.5)	—
HH-I	Indomethacin, 15.0	+	5.77 (5.57–5.97)	10.97 (10.29–11.64)	25.6 (25.1–26.0)	84
HH-I	Phenylbutazone, 75.0	0	2.36 (2.30–2.41)	2.04 (1.76–2.31)	8.1 (7.8–8.3)	—
HH-I	Phenylbutazone, 75.0	+	6.92 (6.76–7.08)	10.91 (10.81–11.00)	21.0 (20.5–21.4)	86
HH-I	Cortisol, 1.0	0	2.44 (2.42–2.45)	4.49 (3.75–5.23)	7.8 (6.5–9.0)	—
HH-I	Cortisol, 1.0	+	5.48 (5.23–5.72)	10.84 (9.22–12.46)	24.3 (20.7–27.9)	82

concentration used, with very little additional suppression above 0.05 μg/ml. In a similar study carried out in the presence of serum, clear suppression of activation was noted at 0.05 μg cortisol/ml, and peak suppression occurred at about 1.0 μg/ml. These data are of particular interest since the biologic regulatory action occurs at physiologic steroid concentrations (23). Synovial fluid from rheumatoid patients contained 0.07 μg 17-hydroxy-corticosteroids/ml of fluid, of which 0.008 μg/ml was unbound cortisol (23).

Dose-response studies for a group of nonsteroidal antirheumatic drugs are shown in Figure 3. It is clear from this study that indomethacin and meclofenamic acid suppress activation in very low concentrations, whereas substantially higher concentrations of phenylbutazone and sodium salicylate were required for suppression in this particular assay. It appeared that once an optimal drug concentration had been reached, further increase in concentration led to no additional suppression of activation.

Direct Exposure of CTAP to Antirheumatic Drugs. The possibility that antirheumatic drugs might actually alter the CTAP added simultaneously to the assay culture was examined by incubating various agents

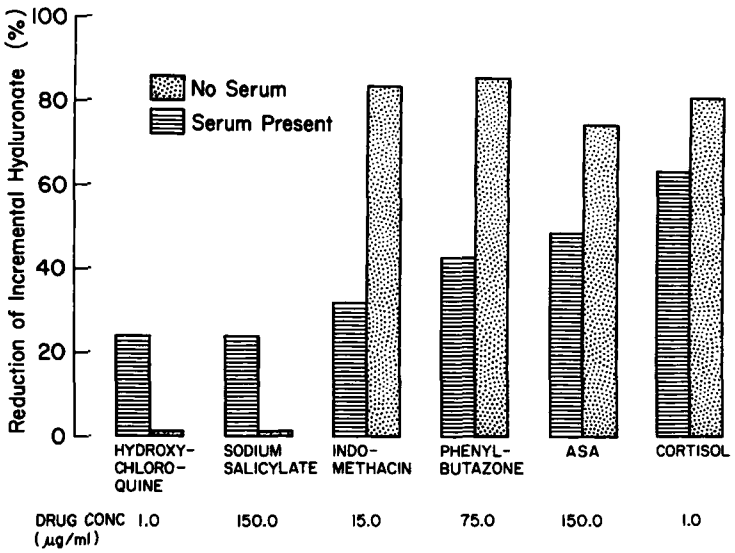

Fig 1. Suppression of connective tissue activation by antirheumatic drugs; the effect of serum. Hydroxychloroquine and sodium salicylate show little antiactivation capacity, and serum depresses that capacity for the more potent agents.

with CTAP for 4 hours at 37° C and then removing the drug by dialysis for 16 hours at 6° C. As shown in Table 4, there was little convincing evidence that direct exposure of CTAP to these drugs resulted in alteration of the activation capacity of the CTAP, particularly in the case of cortisol, hydroxychloroquine and acetylsalicylic acid. While it is possible that indomethacin and phenylbutazone may have actually produced partial alteration of CTAP, we did not rule out the possibility that some of the drugs may have remained bound to CTAP and were carried into the assay culture, thus causing the minimal reduction in CTAP activity shown in Table 4.

The Effect of Time of Drug Exposure on the Antiactivation Effect. It may be seen from Table 5 that most antirheumatic drugs were equally effective if given at either zero time or 2 hours after CTAP was added to the assay cultures. Several drugs were ineffective if

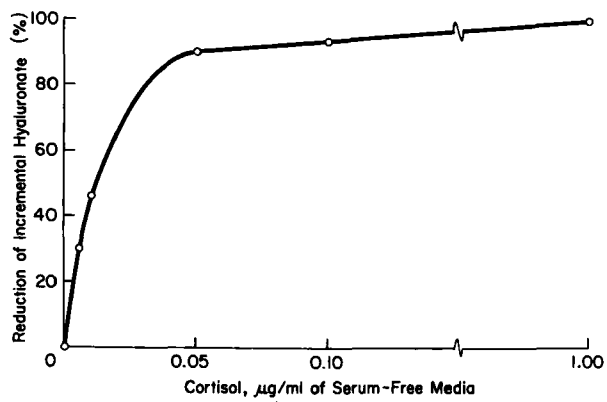

Fig 2. Cortisol suppression of synovial cell activation; dose-response study. Regulation of synovial cell activation occurs at physiologic cortisol concentrations.

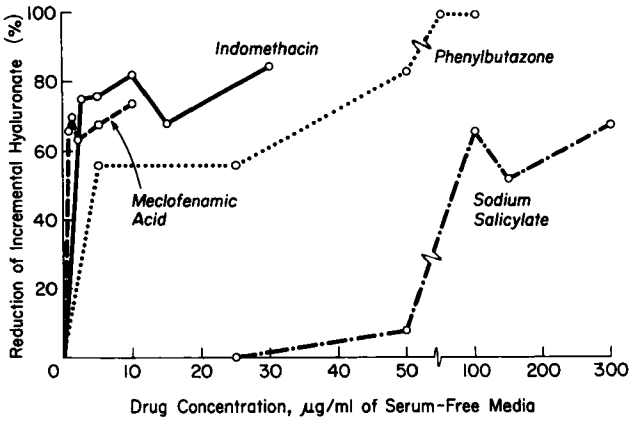

Fig 3. Effect of nonsteroidal agents on connective tissue activation; dose-response study. Nonsteroidal agents tend to show maximal antiactivation activity at concentrations similar to those achieved in clinical use.

Table 4. Drug Alteration of CTAP

Drug exposure* (µg/ml)	Activation induced by drug-exposed CTAP (%)
None	100
Cortisol, 1.0	100
Hydroxychloroquine, 2.0	92
Sodium salicylate, 150.0	87
Indomethacin, 15.0	62
Phenylbutazone, 75.0	73
Acetylsalicylic acid, 300.0	100

*Drug + CTAP incubated 4 hours at 37°C, then dialyzed 16 hours at 6°C.

added as late as 8 hours after CTAP, and very few had a measurable effect on the activation process if given 24 hours after CTAP stimulation of the cultures—ie, after the activation sequence was fully established.

DISCUSSION

Conceptualization of antiinflammatory drug action is made difficult by the complexity of the inflammatory process (depicted schematically in Figure 4). Acute inflammation embraces the phenomena associated with alterations of the microcirculation, the exudative process and tissue destruction associated with anoxia or the release of lysosomal enzymes. The dominant cells in this early reaction are largely derived from the leukocytes of the circulating blood. Chronic inflammation includes the proliferative (reparative or destructive) reaction, predominantly reflecting the performance (or malfunction) of connective tissue cells. It is clear from Figure 4 that several pathways lead to the adverse functional and anatomic changes resulting from chronic inflammation. A particular antiinflammatory agent may exert no inhibitory capacity on some pathways, and show a range of suppressive ability against the others. In this context, the performance of a drug against chronic inflammation would depend *not only on how effectively it inhibited particular pathways, but also on the relative importance of the different pathways to the perpetuation of the inflammatory process* in a given clinical situation. We presently have no satisfactory methods for weighing the importance of the different components of the inflammatory process outlined in Figure 4, either in terms of their proportional contributions to tissue dysfunction or destruction, or even their essentiality to the self-perpetuating character of chronic inflammation.

The present study examines the in vitro action of several antirheumatic drugs against a portion of the connective tissue component of the inflammatory process and may provide a

Table 5. Time of Drug Exposure: Effect on Activation

Drug	Conc (μg/ml)	Reduction in incremental hyaluronate (%)* $T_{0\,hr}$ Transcription	$T_{2\,hr}$ Translation	$T_{8\,hr}$	$T_{24\,hr}$ Specific product formation
Flufenamic acid	4.0	100	100	100	59
Mefenamic acid	80.0	100	100	100	73
Meclofenamic acid	5.0	65	75	0	0
Phenylbutazone†	75.0	100	100	66	65
Phenylbutazone‡	75.0	82	82	43	0
Indomethacin	15.0	53	64	0	0
Hydroxychloroquine	2.0	28	25	0	0
Sodium salicylate	150.0	38	60	15	0
Acetylsalicylic acid	150.0	63	63	0	0
Cortisol†	1.0	85	80	58	0
Cortisol‡	1.0	89	71	67	0

*T indicates the number of hours following addition of CTAP to cultures when a particular antirheumatic agent was added. The arrows under *Transcription*, *Translation* and *Specific product formation* are meant to convey, in a schematic fashion, an impression of the temporal sequence and duration of these processes.

†Assay strain = HH

‡Assay strain = ArM

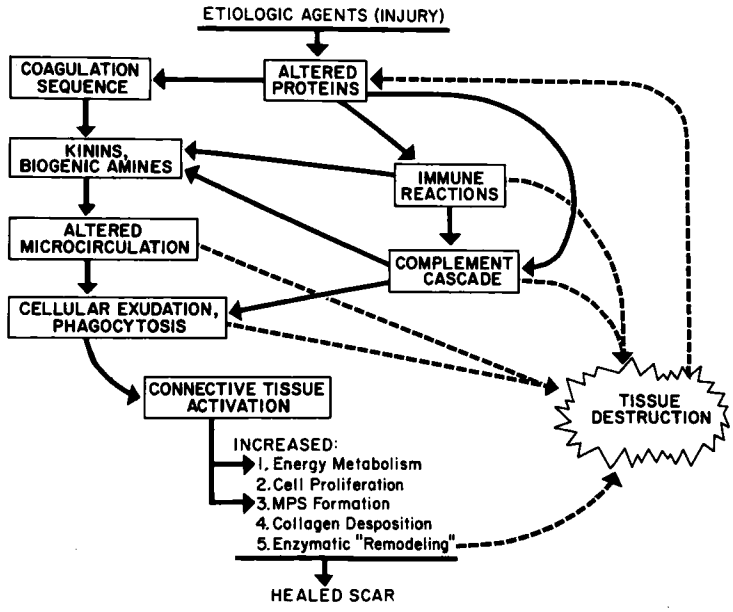

Fig 4. A schematic diagram of the interrelationship of major systems involved in the chronic inflammatory reaction.

novel insight into their mechanism of action. Earlier studies indicated that the biosynthesis of connective tissue ground substance components was modified by antirheumatic drugs. Both corticosteroid and nonsteroid antiinflammatory drugs have been reported to depress the biosynthesis of sulfated mucopolysaccharides in cartilage, cornea and heart valve (24, 25). Other data suggest that blockade of sulfate incorporation in these tissues might be the major drug action rather than inhibition of macromolecule biosynthesis (26). Glucocorticoids suppress formation of hyaluronate (a nonsulfated mucopolysaccharide) by approximately 50% in human synovial cells studied in a basal (ie, nonactivated) state in tissue culture (27, 28). A recent report from this laboratory indicated that indomethacin caused a minor, equivocal depression of hyaluronate formation in basal synovial cultures, and phenylbutazone caused a definite, but minor, reduction in hyaluronate formation (29).

From Table 3 it is clear that hydroxychloroquine, sodium salicylate, acetylsalicylic acid, indomethacin and phenylbutazone exert virtually no depressive effect on the basal hyaluronic acid synthesis rate of human synovial cells. In other experiments meclofenamate (5.0 μg/ml) did not depress basal hyaluronic acid synthesis. It is interesting that cortisol suppression of basal hyaluronic acid synthesis by synovial cultures is most consistently demonstrated in the presence of serum-containing media. On the other hand, when human synovial cells are activated, exposure to corticosteroids and most of the nonsteroidal antiinflammatory drugs results in marked suppression of incremental hyaluronic acid synthesis. These observations suggest that *the major role of antirheumatic drugs in reducing synovial hyaluronate synthesis in pathologic states may not be related primarily to interference with polymer synthesis per se, but instead to interdiction of synovial cell activation.*

In studies reported elsewhere (30), it was demonstrated that synovial cell activation was independent of DNA synthesis, but required DNA-mediated RNA synthesis and protein synthesis. Inhibitors of transcription (actinomycin D, mithramycin, acidine orange, chromomycin A_3 and α-amanitin) and translation (puromycin, cycloheximide and acetoxycycloheximide) all blocked the activation process if added to cultures simultaneously with CTAP. Inhibition of transcription 4 hours after addition of CTAP produced no inhibition of activation, and, similarly, interference with protein synthesis 16 hours or more following an activating stimulus was without inhibitory effect on the activation process. In the context of these observations it was interesting to note that the efficacy of those antirheumatic drugs showing an antiactivation effect was closely tied to the timing of drug administration in relation to the activating event (addition of CTAP). The antirheumatic drugs were most effective if added to cultures simultaneously with CTAP or 2 hours later, early enough to interfere with the transcription component of the activation process. Drugs added 8 hours after the activating event (during translation) frequently showed reduced efficacy in blocking activation, although substantial suppression was usually demonstrable. If 24 hours elapsed after an activating event, most of the antirheumatic drugs were without effect. Drugs suppressing hyaluronic acid synthesis in fully activated cells (24 hours post-CTAP) presumably would act on the polymer synthesizing mechanism itself. Both flufenamic and mefenamic acid partially suppressed incremental hyaluronic acid synthesis in fully activated cells. The possibility of individual cell strain differences in drug sensitivity was raised by the experiments with phenylbutazone where incremental hyaluronic acid synthesis was substantially suppressed in fully activated "HH" cells but not in the "ArM" strain. In summary, *the in vitro evidence suggests that antirheumatic drugs suppress hyaluronate synthesis by synovial cells primarily by interfering with the activation sequence. Further, the time-related experiments*

are consonant with the possibility that the effective drugs act mainly on the transcriptional and translational component of the activation sequence.

Salicylates, the anthranilic acid derivatives, phenylbutazone and indomethacin are known to be capable of uncoupling oxidative phosphorylation in mammalian cells (24), and it has been suggested that these drugs may antagonize the inflammatory process by denying adenosine triphosphate to energy-dependent components of the process. The drug concentrations used here (which mimic those attainable clinically) failed to stimulate glucose uptake or lactate formation. These phenomena *are* demonstrable when synovial cells are exposed to 4×10^{-5} M 2,4-dinitrophenol, an agent whose major action is uncoupling of oxidative phosphorylation (31). This is indirect evidence that uncoupling of oxidative phosphorylation is not a significant factor in antirheumatic drug blockade of activation.

Dose-response studies carried out in a tissue culture system may provide information relevant to extracellular fluid drug concentrations required to modify cell function. It was interesting to note that phenylbutazone and sodium salicylate interfered maximally with the activation process at concentrations similar to those achieved in the blood of patients treated with these agents. Indomethacin, on the other hand, blocked activation in concentrations well below those seen in clinical situations. Cortisol, tested in serum-free medium, exhibited marked antiactivation capabilities even at physiologic concentrations, but higher concentrations were required when serum was present. In this in vitro test situation, serum proteins clearly reduced the antiactivation effects of indomethacin, phenylbutazone, acetylsalicylic acid and cortisol by amounts ranging from 20.0 to 60.0% of the values noted in a serum-free environment. In intact man these agents are known to be bound to plasma proteins, a phenomenon important to the extent that it dictates availability of drug to target cells.

It was not surprising to find that gold salts and antimalarial drugs, known for their delayed onset of action against rheumatoid inflammations, had little or no activity against the activation process. These agents may modify the kinetics of lysosomes and/or their enzymatic contents, possibly interfering directly with some of the tissue destructive pathways.

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