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Effect of glucocorticoids on the hexose monophosphate pathway in human rheumatoid synovial lining cells *in vitro* and *in vivo*

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SUMMARY Human rheumatoid synovial lining cells have up to four times the capacity to oxidize glucose 6-phosphate, the first step of the hexose monophosphate pathway, as do the nonrheumatoid cells. The reducing equivalents produced by this system have many significant metabolic effects. Exposure of these cells to 10^{-5}M prednisolone *in vitro*, or to 6 mg/day *in vivo*, causes some depression of this activity in the rheumatoid synovial lining cells; less than this dose of steroid, or the administration of nonsteroidal drugs *in vivo*, has little or no effect. The depression of activity produced by 6 mg/day does not bring this activity down to the value found in nonrheumatoid synoviocytes.

Even though synthetic glucocorticoids, such as hydrocortisone and prednisolone, have been much used in the treatment of rheumatoid arthritis, their mode of action is not fully known. Attention has been paid to the possible beneficial effect of such steroids on lysosomes either in the inflammatory cells or in the cells of the synovial tissue because it is known that this type of steroid stabilizes the membranes of isolated lysosomes (Weissmann, 1968, 1969). Bitensky *et al.* (1974) showed that a sufficient concentration of 10^{-4}M glucocorticoids applied to synovial tissue maintained *in vitro* produced some stabilization of the lysosomal membranes in the synovial lining cells. Moreover they showed a quantitatively similar stabilization *in vivo* in patients who had been receiving at least 5 mg prednisolone a day; lower single daily doses of prednisolone had little or no effect. However, the maximum degree of stabilization achieved by even the higher concentrations of glucocorticoid was still only 30–50% of that found in the relatively normal synovial lining cells of nonrheumatoid joints.

It has also been observed (Butcher *et al.*, 1973) that the activity of glucose 6-phosphate dehydrogenase in rheumatoid synoviocytes was three to four times greater than in the synoviocytes of nonrheumatoid synovial tissue. Because the hexose monophosphate pathway, and the reducing equivalents generated by it, subserve many functions in cellular metabolism, the raised activity of glucose 6-phosphate dehydrogenase, the first step in this pathway, indicated a significant difference in the metabolic activity in the rheumatoid synoviocytes. Moreover there is evidence that cortisone, and steroids of similar configuration, influence the production and utilization of reducing equivalents generated by glucose 6-phosphate dehydrogenase activity (Chayen *et al.*, 1974). We therefore decided to investigate whether glucocorticoids affected this abnormally high rate of generation of NADPH, by glucose 6-phosphate dehydrogenase activity, in human synovial lining cells.

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Material

The study was made on 13 specimens from 10 patients who had been treated either with nonsteroidal drugs or with <5 mg prednisolone/day; 8 specimens from 7 patients who had received 5 mg prednisolone or more each day; and 1 specimen from a patient treated with Acthar gel (Table 1). All had definite or classical rheumatoid arthritis according to the diagnostic criteria of the American Rheumatism Association (Ropes *et al.*, 1959). The specimens were obtained at synovectomy. All patients who had been treated with prednisolone were given 100 mg

Table 1 *Patients studied*

Those treated with <5 mg prednisolone/day, or with nonsteroidal drugs only			*Those treated with at least 5 mg prednisolone/day, or with ACTH*			
Specimen no.	*Sex*	*Age (yrs)*	*Specimen no.*	*Sex*	*Age (yrs)*	*Duration of therapy (yrs)*
1029	M	60	1078	F	59	4
1052	F	50	1085	F	62	5
1057			1089			
1080	F	58	1205	F	65	5
1084			1267	M	78	3
1212	F	43	1339	F	29	2 (ACTH)
1213			1373	M	55	12
1233	F	54	1445	F	45	4
1260	F	55	1462	F	69	3
1345	F	48				
1430	F	46				
1503	F	35				
1510	F	50				

hydrocortisone with the premedication immediately before operation. In addition, 4 specimens of non-rheumatoid synovial tissue, obtained during routine arthrotomy for internal derangements, were used for *in vitro* studies.

Samples including the synovial lining, approximately 5 × 3 × 3 mm, were chilled by precipitate immersion into *n*-hexane (B.D.H. 'free from aromatic hydrocarbon' grade, b.p. 67–70°C) at −70°C for up to 1 min. They were then stored in a dry tube at this temperature for up to 3 days. Other specimens were cut into similar-sized pieces and maintained *in vitro* in Trowell (1959) maintenance culture for 20 hours (Poulter *et al.*, 1970). Various concentrations of glucocorticoids were added to the Trowell T8 medium used for maintaining some of these specimens. Where water-insoluble steroids were used, these were dissolved in absolute ethyl alcohol and added to the T8 medium to achieve a final concentration of alcohol of less than 1%. In such cases an equivalent concentration of alcohol was included in the control. At the end of the culture period the specimens were chilled and stored in the same way as the biopsy specimens.

Methods

The tissue was sectioned at 12 μm in a Bright's cryostat with the cabinet temperature of −25°C and the knife cooled by having its haft packed with solid carbon dioxide. The sections were tested by the methods of Chayen *et al.* (1973a) for the following activities: (i) for glucose 6-phosphate dehydrogenase activity (total generation of NADPH), or (ii) for maximal oxidation of NADPH, or (iii) for the amount of NADPH, generated within the section by glucose 6-phosphate dehydrogenase activity, which was oxidized by the cells. For (i) the intermediate hydrogen-carrier phenazine methosulphate (PMS) was included in the reaction medium. Other studies have shown that this quantitative cytochemical method yields the same amount of dehydrogenase activity as the more conventional biochemical estimation (Altman, 1972). For (ii) the substrate was exogenously added NADPH, while for (iii) it was glucose 6-phosphate and $NADP^+$ with no intermediate hydrogen-carrier so that the reduction of the tetrazolium salt, by endogenously formed NADPH, depended solely on the microsomal respiratory pathway of the cells themselves (Chayen *et al.*, 1973b).

The reaction media contained 3 mg/ml of purified neotetrazolium chloride (Merck) in 0·05 M glycyl glycine buffer, pH 8·0, containing 20% w/v polyvinyl alcohol (PVA: BO5/140; Wacker, Germany); 3 mM $NADP^+$ and 5 mM glucose 6-phosphate disodium salt (Boehringer); for measuring the dehydrogenase activity the medium also contained 0·2 mg/ml PMS (Sigma). Before use, the medium was saturated with nitrogen and equilibrated to 37°C.

The end-product of these reactions was the highly coloured and precipitated formazan (reduced neotetrazolium chloride). This was measured in each of a number of synovial lining cells by means of a scanning and integrating microdensitometer.

Results

REPRODUCIBILITY OF MEASUREMENTS

Two specimens of synovial tissue were obtained from the joint in 5 subjects. The results (Table 2) give a measure of the sampling error which can occur between different parts of one synovial tissue. The mean value for all the readings from each specimen differed from the mean of all the readings (both specimens) by up to ±19%, with three of the values differing by less than ±5%. The reproducibility between serial sections taken from any one specimen

Table 2 *Reproducibility of glucose 6-phosphate dehydrogenase activity (integrated extinction × 10^3/unit field per 10 min)* in different samples from the same joint*

Specimen no.	*Section no.*		*Mean*	*Total variation (as % of mean)*
	1	*2*		
1029	418	411	415	1
1052	424	525	474	11
1080	468	433	450	4
1212	382	406	394	3
1085	179	120	150	19

* Activities in Tables 3–6 were also measured in this way.

was investigated in 8 specimens (Table 3). The reproducibility was within ±10%; the standard error of the means of all readings was usually considerably better than this (as shown in Tables 4, 5, 6).

EFFECT OF GLUCOCORTICOIDS IN VITRO

In general the oxidative activities were well maintained after 20-hour maintenance culture (Table 4). In the nonrheumatoid tissue (Table 4) hydrocortisone at 10^{-4}M concentration produced some depression of the glucose 6-phosphate dehydrogenase activity (tested with PMS). In specimen 1152, 10^{-6}M hydrocortisone produced no change but an appreciable effect was found with 10^{-5} mol/l. This effect of 10^{-4}M hydrocortisone on this activity in synovial lining cells from rheumatoid joints was much more marked (Table 4). Some depression of activity was observed even at 10^{-6} mol/l (specimen 1430). This depression of the dehydrogenase activity was mimicked to some extent by a small diminution in the rate of oxidation of the NADPH which was generated from this enzyme activity. However, the high residual activity of this enzyme makes it unlikely that this was rate-limiting. The diminution in oxidation of exogenously added NADPH makes it more likely that it was this microsomal respiratory pathway which was affected. However, these effects were very small relative to the depression of the primary dehydrogenase activity (production of NADPH in the presence of PMS).

EFFECT OF GLUCOCORTICOIDS IN VIVO

In biopsies of rheumatoid synovia from patients treated only with various nonsteroidal anti-inflammatory drugs, the lowest activity of glucose 6-phosphate dehydrogenase of the synovial lining cells was 376 units; the values ranged up to 560 units (Table 5). Similar levels of activity were found in patients who had received 2·5 or 3 mg prednisolone a day, but one patient receiving 4 mg a day did show some apparent suppression of this activity although the ability to oxidize NADPH (first 2 columns of Table 5) remained unaltered, at least in these samples.

In contrast (with one exception), in patients receiving at least 5 mg prednisolone a day or 8 units/day Acthar gel, the activity of glucose 6-phosphate dehydrogenase was never above 380 units, most values being under 200 units of activity (Table 6). The ability to oxidize exogenously added NADPH (column 2, Table 6) did not show any consistent response compared with that found for the other patients (Table 5).

The difference in glucose 6-phosphate dehydrogenase activity in patients receiving at least 5 mg prednisolone a day, as against those receiving less than this dose (i.e. last column in Tables 5 and 6 respectively) was highly significant ($P<0·001$).

Table 3 *Reproducibility of the measurement of glucose 6-phosphate dehydrogenase activity in duplicate sections from the same specimen*

Specimen no.	*Section no.*		*Mean*	*Total variation (as % of mean)*
	1	*2*		
1067	214	212	213	0·4
1085	173	184	178	3·3
1116	218	187	203	7·7
1135	109	100	104	4·3
1212	397	366	382	4·1
1213	405	334	370	9·6
1233	384	454	419	8·2
1445	416	498	457	8·9

Table 4 *Effect of glucocorticoids on synovial lining cells* in vitro *(mean±SEM)*

Specimen no.	*Condition*	*Biopsy or culture condition*	*Oxidation of endogenously generated NADPH*	*Oxidation of exogenous NADPH*	*Glucose 6-phosphate dehydrogenase activity*
1067	Non-Ra	Biopsy	19·6±0·7	29·4±1·1	213·0±4·5
		Culture	19·4±0·9	28·4±1·9	191·0±4·0
		Culture+ 10^{-4}M HC	15·7±1·3	27·4±1·6	170·0±5·3
1116	Non-RA	Biopsy	—	—	203·0±10·3
		Culture	—	—	140·0±6·8
		Culture+ 10^{-4}M ws HC	—	—	120·0±8·0
1135	Non-RA	Culture	13·2±0·5	32·0±1·2	109·0±6·7
		Culture+ 10^{-4}M ws HC	—	25·7±0·8	72·5±2·3
1152	Non-RA	Biopsy	13·1±0·6	33·0±1·2	148·0±6·6
		Culture	14·0±0·6	33·7±1·3	134·0±6·8
		Culture+ 10^{-6}M ws HC	12·3±0·7	27·0±0·7	132·0±8·5
		Culture+ 10^{-5}M ws HC	12·2±0·5	23·4±0·7	104·0±4·2
1212	RA	Biopsy	34·3±1·0	33·9±1·0	382·0±11·9
		Culture	34·0±1·3	32·6±1·1	346·0±11·4
		Culture+ 10^{-4}M HC	26·4±2·0	26·2±1·2	189·0±14·6
1430	RA	Culture	39·6±2·2	33·7±1·6	401·5±14·8
		Culture+ 10^{-6}M prednisolone	38·8±2·0	28·9±2·9	366·7±14·1
		Culture+ 10^{-5}M prednisolone	18·8±0·9	29·9±1·2	240·0±18·5

HC=alcohol-soluble hydrocortisone; ws HC=water-soluble hydrocortisone; — =not measured; RA=rheumatoid arthritis.

Table 5 *Pentose-shunt oxidative activity (mean±SEM) in synovial lining cells of patients treated with <5 mg prednisolone/day*

Specimen no.	*Treatment*	*Oxidation of endogenously generated NADPH*	*Oxidation of exogenous NADPH*	*Glucose 6-phosphate dehydrogenase activity*
1029	No steroids	23·2±1·0	24·4±1·3	418±14·0
1052	No steroids	37·2±1·3	33·5±0·8	424±14·3
1057	No steroids	46·0±2·0	46·2±0·9	525±10·0
1080	2·5 mg/day prednisolone	34·7±0·3	34·9±0·3	471± 5·9
1084	2·5 mg/day prednisolone	34·3±0·3	34·1±0·3	432±14·7
1212	No steroids	34·3±1·0	33·9±1·0	382± 9·3
1213	No steroids	38·0±1·3	37·0±1·3	376±15·0
1233	2·5 mg/day prednisolone	36·7±7·5	36·4±9·1	419±12·8
1260	No steroids	34·2±1·3	33·5±1·0	444±15·5
1345	4 mg/day prednisolone	38·0±1·5	35·0±1·1	230± 7·1
1503	No steroids	—	—	560± 9·4
		—	—	535±10·1
1510	No steroids	—	—	481±11·7
				438±65 (Mean±SD)

Table 6 *Pentose-shunt oxidative activity (mean±SEM) in synovial lining cells of patients treated with at least 5 mg prednisolone/day*

Specimen no.	*Treatment*	*Oxidation of endogenously generated NADPH*	*Oxidation of exogenous NADPH*	*Glucose 6-phosphate dehydrogenase activity*
1078	6 mg/day prednisolone	43·2±2·4	47·1±2·4	378±17·3
1085	,,	17·2±1·1	32·6±1·5	179± 6·3
1089	,,	13·2±0·5	35·5±2·1	120± 5·5
1205	,,	19·8±0·8	25·0±1·1	189± 9·4
1267	3 mg tds prednisolone	14·3±0·1	30·1±0·3	140± 4·2
1339	Acthar gel (Armour) 8 units/day	22·5±0·9	55·0±1·6	109± 6·0
1373	6 mg/day prednisolone	20·5±0·6	20·0±0·7	158± 7·4
1445	3 mg bd prednisolone	—	—	445±13·4
1462	5 mg/day prednisolone	16·4±0·5	20·0±0·3	290± 9·0
				223±120 (Mean±SD)

Discussion

The synovial lining cells of rheumatoid joints differ from those of nonrheumatoid joints in two cellular biochemical aspects. The first is that the lysosomal membranes of the former are considerably more permeable than the latter as tested by quantitative cytochemical methods (Chayen *et al.*, 1971; Chayen and Bitensky, 1971). This pertains even when the synovium is taken from recently traumatized joints in which the lysosomal permeability, as assessed by this test, is greater than in more quiescent joints (Chayen *et al.*, 1971). The second is that the maximal rate of production of NADPH, from NADP, is between 3 and 4 times greater in the rheumatoid than in the nonrheumatoid lining cells (Butcher *et al.*, 1973). It is well known that certain steroids, notably the glucocorticoids, may influence the permeability of membranes, particularly of lysosomes. Moreover, it has been shown that glucocorticoids administered *in vivo* exert some stabilizing influence on the lysosomes of the lining cells of the joints of rheumatoid patients treated with at least 5 mg glucocorticoid per day (Bitensky *et al.*, 1974). However, even though there is good evidence that steroids can influence the activity of purified glucose 6-phosphate dehydrogenase (Marks and Banks, 1960; Levy *et al.*, 1966) and activity of the enzyme in certain tissues (Chayen *et al.*, 1974), there has been no indication that glucocorticoids used in treating rheumatoid arthritis could influence this system.

Our results show that the administration of glucocorticoids to rheumatoid patients is associated with a depression of glucose 6-phosphate dehydrogenase activity, provided that at least 5 mg prednisolone is given daily. At lower dosage the effect is indeterminate or not present. It must be borne in mind, however, that this threshold level may apply only to those joints with refractory disease since it is only these patients who come to synovectomy. For all that, it is of interest that Chamberlain and Keenan (1976) in a double-blind trial showed that patients receiving 3 mg prednisolone/day sustained little clinical benefit whereas some clinical improvement was apparent in those treated with 5 mg/day.

From the studies of Peterson and Wyngaarden (1956) it seems that steroids such as prednisolone become distributed throughout the extracellular

body fluids. Consequently, taking the volume of these fluids as between 11 and 17 litres, 5 mg prednisolone would achieve a concentration outside the tissues of roughly 10^{-6} mol/l. It is noteworthy that in the studies on synovial tissue maintained *in vitro* (Table 4) hydrocortisone appreciably depressed glucose 6-phosphate dehydrogenase activity when the hydrocortisone was present at a concentration of 10^{-5} mol/l. The longer time that prednisolone would act *in vivo* might account for this ten-fold difference in concentration required to achieve the same effect *in vitro* as *in vivo*. Moreover, the duration of glucocorticoid concentration in the body may also influence the effect. Thus in the patient (Table 6, specimen no. 1267) who received 3 mg prednisolone three times a day glucose 6-phosphate dehydrogenase activity was only 140 units of activity, whereas in the patient (Table 6, specimen no. 1445) who received 3 mg twice a day the activity was 445 units, comparable to the values found in Table 5. It may be noteworthy that treatment with Acthar gel (Table 6, specimen no. 1339) produced the lowest activity recorded for any rheumatoid specimen. Perhaps the efficacy of glucocorticoid therapy in depressing the high glucose 6-phosphate dehydrogenase activity found in rheumatoid synovial lining cells depends (a) on the level achieved at any one time, and (b) on the duration of the daily exposure to raised levels of the steroid.

The implication of these findings is that, in unit time, rheumatoid synovial lining cells are capable of producing up to 4 times as many reducing equivalents, associated with glucose 6-phosphate dehydrogenase and NADP in the cytosol, as the non-rheumatoid cells. Yet their rate of oxidation of endogenously or exogenously added NADPH is reasonably similar. One effect of glucocorticoids is to diminish this rate of production of reducing equivalents. However, it is evident that even a dose of 6 mg/day does not depress the glucose 6-phosphate dehydrogenase activity to nonrheumatoid levels.

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