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Effect of glucocorticoids on lysosomes in synovial lining cells in human rheumatoid arthritis

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It is now generally conceded that lysosomal instability in tissues of the rheumatoid joint play some part in the rheumatoid process either directly (*e.g.* Weissmann, 1966, 1967; Page Thomas, 1967, 1969; Chayen and Bitensky, 1971) or by means of an immunological response (Hollander, McCarty, Astorga, and Castro-Murillo, 1965; Weissmann, 1966, 1967; Page Thomas, 1969). Although the critical site of the altered lysosomes was originally sought in the free cells of the synovial fluid, the work of Ball (1968) indicated that the primary site was in the synovial reflexions of the joint. This view gained support from the study by Muirden and Mills (1971), who found that there was no correlation between the degree of joint damage and the amount of lymphocytic infiltration in 42 joints examined. On the other hand there was a significant correlation between the amount of lining cell proliferation and joint damage. Moreover, Muirden (1972) found that high levels of lysosomal enzymes were found in joints in which the histology was dominated by the proliferation of synovial lining cells.

Glucocorticoids such as hydrocortisone or related synthetic analogues have been much used in the treatment of rheumatoid arthritis. Their mode of action, in this context, is not entirely clear. There is good evidence that such corticosteroids can stabilize the membranes of isolated lysosomes (Weissmann, 1968, 1969; Allison, 1968) and some evidence that they may act similarly when applied to living cells (Weissmann, 1969). Lewis and Day (1972) adduced evidence that corticosteroids may stabilize the lysosomes of the free cells in synovial fluid; they presented indirect evidence that a similar effect pertained in rheumatoid synovial tissue but they were not able to show in which cells this response occurred.

In view of these considerations it seemed reasonable to examine the effect of natural and synthetic glucocorticoids on the lysosomes of human synovial lining cells specifically, separate from their effect on lysosomes of the mass of synovial tissue with its very heterogeneous population of diverse cell types. This can be achieved by microdensitometry of the quantitative cytochemical reactions of the lysosomes, the synovial lining cells being measured separately from the other cell types by optical means (Chayen, Bitensky, Butcher, and Cashman, 1971; Bitensky, Butcher, and Chayen, 1973). The effect of the steroids could be investigated in synovial biopsies from patients treated with glucocorticoids. To confirm that any observed effects were due to the direct action of these steroids, it was also possible to study their effects *in vitro*, because it has now been demonstrated that human synovial tissue can be maintained in non-proliferative culture in such a way that, in all respects studied, it is structurally and chemically identical with the tissue at the time of the biopsy (Poulter, Bitensky, Cashman, and Chayen, 1970; Chayen, Bitensky, Butcher, and Cashman, 1973).

Material

Thirteen specimens were examined from twelve patients who had been treated with at least 5 mg./day prednisolone, one specimen from a patient treated with Acthar gel, and fifteen specimens from thirteen patients who had been treated with 4 mg./day or less, or who had not been treated with glucocorticoids at all (Table I, overleaf). All had 'definite' or 'classical' rheumatoid arthritis, according to the diagnostic criteria of the American Rheumatism Association (Ropes, Bennett, Cobb, Jacox, and Jessar, 1959). These specimens were obtained at synovectomy.

In addition, some specimens of non-rheumatoid synovial tissue were obtained during routine arthrotomy for internal derangements.

Selected pieces (not larger than $5 \times 3 \times 3$ mm.) of the specimens were chilled by precipitate immersion at $-70°C$. in *n*-hexane (B.D.H. 'free from aromatic hydrocarbon' grade) for up to 1 min. before being stored in a dry tube at $-70°C$. Other specimens were cut into small pieces and maintained *in vitro*, in Trowell maintenance culture (Trowell, 1959; Poulter and others, 1970) for 20 to 24 hrs. 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 added dissolved in alcohol to

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Table I *Specimens and patients studied*

(a) *Those treated with less than 5 mg./day prednisolone or only with non-steroidal drugs*			(b) *Those treated with at least 5 mg./day prednisolone or with ACTH*			
Specimen no.	*Sex*	*Age (yrs)*	*Specimen no.*	*Sex*	*Age (yrs)*	*Duration of therapy (yrs)*
1029	M	60	1022	F	50	9
1052, 1057	F	50	1078	F	59	4
1080, 1084	F	58†	1085, 1089	F	62	5
1128	F	58	1205	F	65	5
1176	F	63	1267	M	78	3
1182	F	52†	1298	F	60	3
1211	F	43	1329	M	50	7
1233	F	54†	1339	F	29	2 (ACTH)
1260	F	55	1349	M	42	4·5
1345	F	48†	1373	M	55	12
1429	F	46	1445	F	45	4
1503	F	35	1462	F	69	3
1510	F	50				
1212*	F	43				
1430*	F	46				

* Used for *in vitro* studies only.
† Treated with less than 5 mg./day prednisolone.
All patients treated with prednisolone were given 100 mg. hydrocortisone with the premedication immediately before operation. This was too short a time for the hydrocortisone to achieve an effect on the synovial cell lysosomes.

achieve a final concentration of 1 per cent. alcohol in the T8 medium. In such studies 1 per cent. of alcohol was added to the medium used for the 'control' tissue. After maintenance culture these specimens were chilled and stored as were the biopsies.

Methods

The chilled tissues were sectioned at 10 μm. in a Bright's cryostat with the cabinet temperature at −25°C. and with the knife cooled by having its haft packed around with cardice. The unfixed sections were subjected to the quantitative cytochemical test for lysosomal naphthylamidase activity with and without acid pretreatment for testing the stability of the lysosomal membranes, as discussed fully by Chayen and others (1971) and by Bitensky and others (1973). Other sections were tested by the acid phosphatase test for lysosomes and lysosomal fragility (Bitensky, 1963; Bitensky and others, 1973). All the cytochemical methods have been described in detail by Chayen, Bitensky, Butcher, and Poulter (1969) and Chayen, Bitensky, and Butcher (1973). The naphthylamidase reaction was measured in the synovial lining cells selectively by means of a Barr and Stroud GN2 scanning and integrating microdensitometer, with a ×100 oil immersion objective; this gave a scanning spot equivalent to a spot 0·25 μm. diameter in the specimen, as has been shown to be essential for accurate measurement of coloured reaction products dispersed in small particles (Bitensky and others, 1973; Butcher, 1972).

It has been shown (Chayen, Bitensky, and Ubhi, 1972) that the mean activities of triplicate samples measured for lysosomal naphthylamidase activity agreed within ±2 per cent. Since the bound activity represents the subtraction of the free from the total activity, each value having its own standard error, the measurement of bound activity can have an error of as great as ±18 per cent. Thus a value of 20 per cent. bound activity may be 16·4 or 23·6 per cent.; a value of 5 per cent. bound activity may be 4 or 6 per cent.

Results

Effects of glucocorticoids in vitro

The result of maintaining non-rheumatoid synovial lining cells *in vitro* for up to 24 hrs in the presence of hydrocortisone (even at 10^{-5} M) was that the lysosomal membranes were either unaffected or were slightly more stabilized (Table II). This effect was most clearly shown by the increased incubation time required to produce a visible acid phosphatase response. With the rheumatoid tissue, both hydrocortisone and prednisolone stabilized the lysosomes of the synovial lining cells when the concentration of these substances was 10^{-4} M; this stabilization was not observed in Specimen 1052.

Effect of prednisolone in vivo

Biopsy material (15 specimens) was examined from thirteen patients who had either been treated with non-steroidal anti-inflammatory drugs, or with prednisolone at a dosage of not more than 4 mg./day. The state of the lysosomes in the synovial lining cells was measured by determining the amount of lysosomal naphthylamidase activity which was latent, *i.e.* the bound activity. Thus the more stable the lysosomal membranes, the greater was the bound (latent) activity of this enzyme. It will be seen (Table III) that eleven out of the fifteen specimens had no bound activity in their synovial lining cells and that

Table II *Effect of corticosteroids on the lysosomes of human synovial lining cells* in vitro

Specimen no.	*Condition*	*Biopsy or culture conditions*	*Acid phosphatase (time in min.)*	*Naphthylamidase (percentage bound activity)*
1067	Non-rheumatoid	Biopsy	40	35
		Normal culture	40	31
		Normal culture + 10^{-4} M HC	60	31
1071	Non-rheumatoid	Biopsy	20	52·3
		Normal culture	30	23·7
		Normal culture + 10^{-4} M HC	60	44·5
1116	Non-rheumatoid	Biopsy	30	—
		Normal culture	30	—
		Normal culture + 10^{-4} M w.s. HC	30–40	—
1152	Non-rheumatoid	Biopsy	30	—
		Normal culture	30	—
		Normal culture + 10^{-6} M w.s. HC	30	—
		Normal culture + 10^{-5} M w.s. HC	60	—
1052	Rheumatoid	Biopsy	—	0
		Culture + 10^{-4} M HC	—	0
1212	Rheumatoid	Biopsy	—	0
		Culture (pH 7·4)	—	0
		Culture + 10^{-4} M w.s. HC	—	21
1430	Rheumatoid	Culture (pH 7·4)	5	5
		Culture + 10^{-6} M prednisolone	5	5·6
		Culture + 10^{-5} M prednisolone	5	8
		Culture + 10^{-4} M prednisolone	15	16

HC = hydrocortisone (alcohol soluble), w.s. HC = water-soluble hydrocortisone, — not tested.

Table III *Effect* in vivo *of prednisolone (less than 5 mg./day) or of non-steroid therapy*

Specimen no.	*Therapy*	*Naphthylamidase activity (percentage bound activity)*
1029	No steroid	0
1052	No steroid	0
1057	No steroid	0
1080	Prednisolone 2.5 mg./day	0
1084	Prednisolone 2.5 mg./day	3
1128	No steroid	0
1176	No steroid	0
1182	No steroid	0
1211	No steroid	0
1233	Prednisolone 2.5 mg./day	0
1260	No steroids	0
1345	Prednisolone 4 mg./day	6·6
1429	No steroid	5
1503	No steroid	4
1510	No steroid	0

Table IV In vivo *effect of prednisolone (at least 5 mg./day) and ACTH*

Specimen no.	*Therapy*	*Naphthylamidase activity (percentage bound activity)*
1022	Prednisolone 5 mg./day	0
1078	Prednisolone 6 mg./day	3
1085	Prednisolone 6 mg./day	17
1089	Prednisolone 6 mg./day	19
1205	Prednisolone 5 mg./day	15
1267	Prednisolone 3 mg. t.d.s.	0
1298	Prednisolone 5 mg./day	0
1329	Prednisolone 5 mg./day	11
1339	Acthar gel (Armour) 8 units daily	15
1349	Prednisolone 5 mg./day	18
1373	Prednisolone 6 mg./day	18
1445	Prednisolone 3 mg. bd	19
1462	Prednisolone 5 mg./day	0

only one had as much as 6·6 per cent.; this patient had been receiving the highest dose of prednisolone (4 mg./day).

Biopsy material (13 specimens) was also investigated directly from another twelve patients all of whom had been treated with at least 5 mg./day of prednisolone (Table IV). Of these thirteen specimens, five had either negligible amounts of bound activity or none. In the other eight specimens, one had 11 per cent. bound activity; the others had 15 to 19 per cent. bound activity. Included in this group was one patient (Specimen no. 1339) who had received Acthar gel instead of prednisolone.

Discussion

In a previous study (Chayen and others, 1971), it was shown that the synovial lining cells from non-

rheumatoid joints (9 patients) had 31 to 57 per cent. bound naphthylamidase activity; the bound activity was reduced to 28 to 40 per cent. in recently traumatized non-rheumatoid joints (7 patients). The biopsy results on non-rheumatoid specimens in the present study (specimens 1067 and 1071 in Table II) agree with these earlier findings. In contrast, in biopsies from patients with rheumatoid arthritis (13 patients), who had not been treated with steroids, Chayen and others (1971) found that nine had no bound naphthylamidase activity in the synovial lining cells and none had more than 5·2 per cent. bound activity. Very similar results have now been presented (Table III) from thirteen other rheumatoid patients who have been treated either with non-steroidal anti-inflammatory drugs, or with not more than 4 mg./day of prednisolone. Thus it seems that such treatment had no appreciable effect on the stability of the lysosomal membranes in the synovial lining cells.

In contrast, the synovial lining cells in the synovial specimens from patients treated with at least 5 mg./day prednisolone showed more than 10 per cent. lysosomal bound activity in eight of the thirteen specimens. Thus it may be assumed that the treatment had a significant effect in these patients, although the amount of bound activity, and thus the lysosomal stability, was still far short of that found even in the recently traumatized joints in the earlier study.

The studies of Peterson and Wyngaarden (1956) indicate that steroids such as prednisolone become distributed through the extracellular body fluids. Consequently, as a rough calculation, a single dose of 5 mg. prednisolone would achieve a concentration of about 10^{-6} M in these fluids whether they have a volume of 17 or of 11 litres. In the study *in vitro* (Table II) hydrocortisone and prednisolone produced appreciable stabilization of the lysosomes when present at 10^{-4} M concentration, acting for only up to 24 hrs. Some response was found with 10^{-5} M prednisolone; the stabilization of the lysosomal membranes (as measured by increased bound activity) appeared to be dose-dependent in that no stabilization was found with lower doses of the steroids. Thus there would seem to be a factor of at least 10 between the effects produced by these steroids *in vitro* and *in vivo*, which is not surprising in view of the relatively short exposure to them in the *in vitro* system.

These studies have therefore confirmed that, at a sufficient dose-level, glucocorticoids administered *in vitro* stabilize the lysosomes of the synovial lining cells. Of greater practical concern are the findings that they do so in most patients given at least 5 mg./day of prednisolone, but they do not stabilize in any of the patients studied who had received less than this dose. Although the latter conclusion is probably correct, these conclusions may apply only to patients with recalcitrant disease. Thus it is possible that treatment with 5 mg./day prednisolone is effective in a greater proportion of patients than is indicated by our results which necessarily are derived from those patients who, despite treatment, have come to synovectomy.

Summary

Lysosomal activity and the stability of lysosomal membranes in the synovial lining cells of human synovial tissue have been measured by quantitative cytochemistry measured specifically in the lining cells by microdensitometry. Thirteen specimens from patients treated with at least 5 mg./day of prednisolone or ACTH have been compared with fifteen specimens from patients treated with either non-steroidal anti-inflammatory drugs alone or with 4 mg./day or less of prednisolone. It has been shown that some stabilization of these lysosomal membranes *in vivo* may be achieved with the higher doses of prednisolone, but that no stabilization of the lysosomal membranes of the synovial lining cells could be detected in those patients who had been treated with 4 mg./day or less of prednisolone. Although these patients form a selected population, namely of those who came to synovectomy despite treatment, two conclusions may be reached. First, that glucocorticoids administered therapeutically to patients, can stabilize lysosomal membranes of synovial lining cells; secondly that the degree of stabilization may depend on the dose administered with low doses possibly having no significant effect.

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