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Evaluation of potential antirheumatic drugs *in vitro* using lymphocytes and epithelial cells. The selective action of indoxole, methyl glyoxal and chloroquine

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Anti-inflammatory steroids and non-steroid drugs rapidly inhibit the incorporation of radioactivity from tritiated uridine and thymidine into the nucleic acid fraction of cultured epithelial cells and lymphocytes obtained from sheep, rats, rabbits and chickens. Acidic (uncoupling) drugs were also found to inhibit protein synthesis in these particular cells, and protein and nucleic acid synthesis in other cells which were steroid-insensitive. DNA synthesis in epithelial cells and lymphocytes was inhibited by methyl glyoxal (pyruvaldehyde). Chloroquine selectively inhibited thymidine incorporation by the epithelial cells. Indoxole was a potent inhibitor of the metabolism of circulating lymphocytes incubated in protein-free media. These findings may indicate why some of these drugs suppress adjuvant arthritis in rats.

SYNTHETIC and natural corticosteroids related to cortisol (hydrocortisone) have proved particularly valuable for the topical treatment of inflammatory conditions in epithelial tissue. It is well known that these anti-inflammatory–antirheumatic steroids, when given systemically, cause involution of the thymus gland in small animals and depress the number of circulating eosinophils in the bloodstream. This suggests that these steroid drugs may suppress inflammatory disease states by virtue of their action on lymphoid cells and certain leucocytes. This supposition is reinforced by recent observations that a form of experimental arthritis in rats, induced by injection of dead mycobacteria in liquid paraffin (so-called "adjuvant arthritis") and which is powerfully suppressed by anti-inflammatory steroids, is also inhibited by extirpation of the lymph nodes (Newbould, 1964) or by administration of an anti-lymphocyte serum (Currey & Ziff, 1966). If lymphoid cells are essential for the development of this type of experimental arthritis, it seems logical to enquire if current anti-inflammatory drugs are able to influence the metabolic activity of these cells, when they are present either in the blood-lymph circulation or contained within lymph nodes and other lymphoid tissues. The purpose of this communication is to report that the incorporation of radioactivity from labelled nucleosides into the nucleic acid fractions of various lymphoid cells and certain epithelial cells, maintained *in vitro*, is rapidly inhibited not only by corticosteroids but also by other anti-inflammatory and anti-arthritic drugs including indoxole (2,3-bis *p*-methoxyphenylindole) and ICI-43,823 (2-butoxycarbonylmethylene-4-oxothiazolidine).

The two latter drugs inhibit the chronic (lymphoid mediated?) stage of adjuvant arthritis in rats (Glenn, Bowman & others, 1967; Newbould, 1965) but ICI-43,823 has no effect upon the initial, acute, local inflammatory response to injected adjuvant. Both the acute and chronic

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stages of this experimental arthritis are inhibited by anti-inflammatory corticosteroids and by several non-steroid anti-inflammatory acids such as phenylbutazone, indomethacin, the fenamic acids and high doses of salicylates (Newbould, 1963; Winter & Nuss, 1966; Graeme, Fabry & Sigg, 1966; Ward & Cloud, 1966).

Experimental

A population of sheep lymphocytes, containing less than 2% of other cells, was obtained by centrifuging the lymph collected from a cannula inserted into the efferent duct draining the popliteal lymph node of conscious Merino or Corriedale ewes. The lymph was collected at room temperature over 24 hr periods in sterile plastic bottles containing approximately 1 mg of heparin (150 I.U.) and 2 mg of penicillin G. Circulating lymphocytes were also obtained from the thoracic duct lymph of conscious albino rats and anaesthetized New Zealand/California white rabbits. Tissue lymphocytes admixed with other cells, were obtained by dispersing finely chopped lymphoid tissues from young rats, rabbits and chickens (thymus, spleen, bursa of Fabricius) in chilled 0·3M sucrose or Krebs-Ringer media with the aid of an all-steel homogenizer: tissue debris was removed by very low speed centrifugation and the cells were collected as the fraction sedimenting above erythrocytes (if present) at 700 $\times$ g for 10 min. Three lines of polyploid epithelial cells, H(uman) EP(ithelial)-2, human amnion ("U" cell) and pig kidney (PK), were obtained from continuous cultures maintained by the Microbiology Department, John Curtin School of Medical Research. These different types of lymphocytes and epithelial cells were washed once in sterile Hank medium and resuspended in a Krebs-Ringer phosphate medium (pH 7·4) containing glucose (7mM) to which heparin (2 IU/ml) was added, to facilitate cell dispersion. Calcium was omitted from the medium in experiments with epithelial cells to minimize cell clumping. The phosphate component was replaced by isotonic Trishydrochloride pH 7·4, for experiments with inorganic [^{32}P]phosphate. The drugs were added as solutions in dimethylformamide or dimethylsulphoxide (final concentration of these solvents was 1% v/v) to aliquots of the cell suspension in round-bottomed centrifuge tubes containing 10–15 $\times$ 10^6 cells/ml, 2 min before addition of radioactive substrates. These mixtures of cells, drugs and substrate were incubated at 37° for 30 min, with slow shaking in air. Incubations were terminated by rapid freezing or by addition of strong acid.

Drug action on these cells was detected by measuring the rates of incorporation of [^{14}C]-labelled amino-acids (an algal protein hydrolysate), [5-^{3}H]uridine and [6-^{3}H]thymidine into material insoluble in 10% (w/v) trichloroacetic acid. All the radioactivity incorporated from added [^{3}H]uridine into the trichloroacetic acid-insoluble fraction was soluble in 10% trichloroacetic acid on heating for 10 min at 95° and high resolution autoradiographs (kindly made by Dr. B. Morris) of washed glutaraldehyde-fixed uridine-labelled sheep lymphocytes indicated that the radioactivity was principally located in the nucleus. The radioactivity incorporated from [^{3}H]thymidine was almost exclusively located in the cell

nucleus and the bulk of this radioactivity was soluble in hot 10% trichloroacetic acid (=nucleic acid). With all the lymphoid cells examined, a persistent thymidine-derived radioactive component (approximately 10–15% total incorporated radioactivity) resisted solution in hot acid and radioactivity was also consistently found in chloroform–methanol (2 : 1 v/v) extracts of well-washed cold trichloroacetic acid-insoluble material. Epithelial cells and micro-organisms incorporated very much less radioactivity (<5% total incorporated) from thymidine into this ("protein") fraction, insoluble in hot acid (cf. Tsien, Duncan & Sheppard, 1967).

Results

Table 1 shows that all the anti-inflammatory anti-arthritic drugs investigated rapidly inhibited incorporation of radioactive uridine into nuclear RNA by "pure" sheep lymphocytes. RNA labelling in other "lymphocytes" (from rat and rabbit thoracic lymph and thymus tissue, or chick spleen, thymus and bursa of Fabricius) was also inhibited by the same concentrations of these drugs, except indoxole (see below). Previous *in vivo* stimulation of sheep popliteal lymph lymphocytes (by subcutaneous injection of swine influenza virus below the popliteal node) greatly enhanced DNA synthesis and, after several days, stimulated protein synthesis

TABLE 1. EFFECT OF ANTI-ARTHRITIC DRUGS, CHLOROQUINE AND METHYL GLYOXAL UPON THE *in vitro* LABELLING OF NUCLEIC ACIDS AND PROTEIN IN LYMPHOCYTES (LC) FROM SHEEP POPLITEAL LYMPH AND IN CULTURED HUMAN CELLS OF EPITHELIAL ORIGIN

Figures are percentage incorporation of (i) [5-^{3}H]uridine into RNA (ii) [6-^{3}H]thymidine into DNA and (iii) mixed [^{14}C]amino-acids into protein, by cells co-incubated with drugs for 30 min at 37°, compared with that in drug-free controls (containing 1% v/v dimethylformamide) and are taken from duplicate experiments.

Drug	Conc. ($\times 10^{-4}$M)	Radioactivity % in Sheep LC**			"HEP-2"			"U-cell"		
		RNA	DNA	Prot.	RNA	DNA	Prot.	RNA	DNA	Prot.
None	—	100	100	100	100	100	100	100	100	100
Indoxole	0·02	65	85	85						
	0·1	30	36	75	90	100	94	90	82	100
	0·5	15	12	40	68	76	83	52	39	80
Bis-2,3-phenylindole	0·1	50	43	35						
	0·5	8	7					75	85	76
ICI-43, 823	2·5	50	61	100	54		80	55		86
	5·0	38	42	92	24		68	27	27	47
2,5-Diphenyloxazole	0·5	38	12	40						
	1·0							38	50	100
Cortisol	2·5	52	70	90	56	55	100	39	35	65
Cortisone	1·0	23	62	96	20	25	95	26	21	80
Cortisol-21-aldehyde	1·0	31	47	65	51	57	63	30	36	67
Cortisol-FPP*	0·25	25	33	80						
	0·5	5		25	19	34	100	7	15	68
Tetrahydrocortisol	2·5	28	42	65	50	44	74	40	32	64
Desoxycorticosterone	1·0	20	48	73	29	24	68	19	12	37
Phenylbutazone	1·0	14	22	62	70	91	81	46	75	74
Indomethacin	1·0	20	38	70	72	93	75	83	47	52
Flufenamic acid	1·0	11	12	17	66	39	34	61	8	13
2,4-Dinitrophenol	1·0	22	61	35	54	65	49	59	16	29
Methyl glyoxal	10	76	10	18	100	29	15	100	53	49
Chloroquine phosphate	10	93	90	84	87	55	92	100	75	94

* FPP = 2′-(*p*-fluorophenyl)-[3,2-C]-pyrazole.

but did not appreciably alter the pattern of drug sensitivity of these circulating cells.

Those drugs that were the most powerful inhibitors of uridine incorporation also significantly inhibited amino-acid incorporation into protein and the incorporation of thymidine into DNA by these lymphocytes, within 30 min. The anti-inflammatory steroids and indoxole (at concentrations indicated in Table 1) had no effect on nucleic acid and protein biosynthesis in spermatozoa, *Tetrahymena pyriformis*, baker's yeast or *Aerobacter aerogenes*, and somewhat variable activity in inhibiting protein and nucleic acid synthesis in three different types of continuously cultured cells of epithelial origin (see Table 1). In PK cells, uridine incorporation, but not protein synthesis, was inhibited more than 50% by 100 μM steroids, >50 μM indoxole and 250 μM ICI-43,823. ICI-43, 823 also inhibited nucleic acid synthesis in spermatazoa, *Tetrahymena* and *Aerobacter*. Anti-inflammatory drugs which uncouple oxidative phosphorylation (Whitehouse, 1965) and 2,4-dinitrophenol, inhibited protein and nucleic acid biosynthesis in all lymphoid and epithelial cells examined and also in other cell types, e.g. spermatazoa, *Tetrahymena*, yeast and *Aerobacter*. Inhibition of protein synthesis in lymphocytes and epithelial cells by these uncoupling drugs (100 μM phenylbutazone, indomethacin and flufenamic acid) was detectable after short incubation periods (1 to 8 min) and was probably not merely a consequence of reduced (messenger) RNA synthesis. Reduction of RNA synthesis by 50% in sheep lymphocytes and human amnion epithelial ("U") cells by actinomycin D (2 μg/ml) had almost no effect on the incorporation of amino-acids into protein within 30 min of drug addition, suggesting that the natural life of the messenger RNA molecules in these particular animal cells was not particularly short (also see Allfrey & Mirsky, 1963).

Relatively high concentrations of cortisol (>100 μM) were required to inhibit RNA synthesis in these short-term experiments with lymphocytes. Less polar steroids such as tetrahydrocortisol, cortisone, corticosterone, desoxycorticosterone, and even 5α-pregnan-3,17,20-triol, were more potent in inhibiting uridine incorporation than cortisol itself. The absolute potency of these individual steroids was diminished, but their relative potency was not altered, when lymph proteins were added to the incubation medium. Prednisolone, cortisol-21-aldehyde (from G. D. Searle and Merck) and cortisol-2'-(*p*-fluorophenyl)-[3,2-C]-pyrazole, FPP (Hirschmann, Steinberg & others, 1963) were notably more potent than cortisol as inhibitors of protein and nucleic acid synthesis in epithelial cells and in lymphocytes obtained from both lymph and the fixed lymphoid tissues.

Indoxole was remarkably potent in suppressing the metabolism of circulating lymphocytes, being effective at 2–5 μM in the absence of protein. At least 10 times these concentrations of indoxole were needed to suppress protein and nucleic acid synthesis in epithelial cells and tissue lymphocytes (from thymus, bursa). Indole and anisole which each represent $\frac{1}{3}$ of the indoxole molecule, singly and together (0·5 mM) had no effect on lymphocyte RNA synthesis. The 2,3-bis-phenylindole analogue showed similar

activity to indoxole but successive replacement of the phenyl groups by methyl groups much diminished the drug acitivity *in vitro*. 2,5-Diphenyloxazole (PPO) was much more potent than ICI-43,823 *in vitro*.

None of the neutral anti-inflammatory drugs listed in Table 1 impaired the uptake or oxidation of [(1,6 or U)-^{14}C] glucose to lactate and CO_2 and of [^{14}C]acetate to CO_2 by sheep lymphocytes at drug concentrations inhibiting uridine incorporation by 60–80%. The "spurious" activity of desoxycorticosterone could however be largely attributed to its effect on energy-yielding reactions in these cells as this particular (non anti-inflammatory) steroid inhibited glucose and acetate oxidation and also depressed the incorporation of inorganic [^{32}P]phosphate into organic phosphates.

Chloroquine phosphate (1 mM), a slow-acting anti-arthritic drug, did not inhibit lymphocyte metabolism in these short-term experiments, but did partially inhibit thymidine incorporation by the epithelial cells and by a protozoon, *Tetrahymena pyriformis*. This parallels its relatively selective effect upon DNA synthesis in chloroquine-sensitive bacteria (O'Brien, Olenick & Hahn, 1966). Chloroquine does not inhibit adjuvant arthritis in rats (Newbould, 1963; Graeme & others, 1966; Ward & Cloud, 1966). This is understandable if this drug is unable to suppress DNA biosynthesis and the consequent proliferation of lymphocytes, following stimulation of lymphoid cells by adjuvant passing into the lymph nodes.

Methyl glyoxal (pyruvaldehyde) which may act as an anti-cancer agent (Szent-Györgyi, Együd & McLaughlin, 1967), selectively inhibited DNA synthesis in these lymphoid and epithelial cells and in *Tetrahymena*. (The apparent suppression of protein synthesis may be the result of combination of this aldehyde with [^{14}C]amino-acids.) This action of methyl glyoxal contrasts with that of cortisol-21-aldehyde, another α-ketoaldehyde, which apparently suppresses both RNA and DNA synthesis in these cells. Neither cortisol-aldehyde (0·5 mM) nor methyl glyoxal (3 mM) uncouples oxidative phosphorylation in rat liver mitochondria (under conditions given by Skidmore & Whitehouse, 1965) which supports the inference that both these ketoaldehydes are relatively selective inhibitors of polymer biosynthesis. Diacetyl (1 mM), the simplest diketo analogue of methyl glyoxal, had no effect on thymidine incorporation by the lymphoid and epithelial cells but actually stimulated uridine incorporation by these cells.

Disussion

Measurements of the incorporation of tritiated uridine and thymidine by lymphoid cells (and by certain lines of epithelial cells) *in vitro* would seem to provide a suitable metabolic system for examining potential antirheumatic drugs, especially since both acidic (uncoupling) and neutral drugs may give a positive response in the one system. In this respect this system has an advantage over other proposed *in vitro* biochemical assays for potential anti-inflammatory activity, which either primarily respond to the acidic drugs (Mizushima & Nakagawa, 1966; Skidmore & Whitehouse, 1966) or respond to steroids only after a long time lag (Whitehouse & Boström, 1962). However, a structure-action relationship

for corticosteroid derivatives obtained from such *in vitro* studies may be misleading as it has been found that all the types of lymphocytes examined, respond both to those steroids which strongly inhibit respiration, e.g. desoxycorticosterone (Jensen & Neuhard, 1961) as well as to those which do not, e.g. cortisol. In this respect these findings are at variance with recent observations upon rat "thymocytes" preincubated with steroids for 3 hr before measuring nucleic acid synthesis (Makman, Dvorkin & White, 1966).

The present findings also indicate that indoxole is probably more cell-specific than the anti-inflammatory steroids and so might possibly be considered a forerunner of a new class of fairly selective drugs, whose action is primarily directed against certain lymphoid cells (in this case, circulating lymphocytes). Such drugs could have important implications for immunobiology, organ transplantation and the treatment of auto-immune diseases. Powerful though they are, the cytotoxic alkylating agents and the currently available anti-inflammatory acidic drugs and steroids do not attain this ideal of selective anti-lymphoid activity.

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