The Influence of Non-steroidal Anti-inflammatory and Antithyroid Agents on Myeloperoxidase-Catalysed Activities of Human Leucocytes

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SUMMARY

Viable leucocytes obtained fresh from normal human subjects were shown to be able to catalyse the *in vitro* iodination of bovine serum albumin (BSA) in a H_2O_2-generating system. The rate and degree of iodination were greatly improved by sonication of the cells. A balanced salt solution was a more favourable medium than phosphate buffer for the myeloperoxidase (MPO)-catalysed iodination of whole cells and sonicated cells. Reactions known to be catalysed by other peroxidases (e.g. thyroid peroxidase (TPO) and lactoperoxidase), such as inorganic iodide exchange for organic iodine in di-iodotyrosine (DIT) and the de-iodination of thyroxine (T_4), were also catalysed by the sonicated leucocyte suspension in the system used.

The non-steroidal anti-inflammatory drugs indomethacin, flufenamic acid and naproxen were far less effective inhibitors of MPO-catalysed BSA iodination of sonicated leucocytes at concentrations expected in blood with therapeutic dose levels than was observed earlier with TPO-catalysed *in vitro* iodination of BSA.

The antithyroid drug methylmercapto-imidazole (MMI) inhibited *in vitro* MPO-catalysed ^{131}I delabelling of ^{131}I-DIT at all concentrations between 10^{-7} and 10^{-2}M, whereas ^{131}I-T_4 delabelling was markedly stimulated at the same drug concentrations. On the other hand, ^{125}I incorporation into ^{131}I-DIT was not affected by increased concentrations of MMI up to 10^{-5}M. At higher drug concentrations the drug caused inhibition of MPO-catalysed exchange of inorganic iodide for organic iodine in DIT.

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Leucocytes possess several mechanisms for destroying invading organisms. Of these bactericidal processes, iodination of invading organisms is perhaps the most important.[1] This iodination reaction has been demonstrated to be affected by myeloperoxidase (MPO) and H_2O_2 generation during phagocytosis.[2] The exceptionally high concentration of MPO in normal leucocytes of not less than 5% of the dry weight of the cell[3] is probably indicative of the importance and efficiency of MPO-catalysed functions of neutrophilic polymorphonuclear leucocytes.

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The iodination reaction of MPO seems to be similar to that of thyroid peroxidase (TPO), which is also H_2O_2-dependent for the oxidation of iodide and subsequent iodination reactions. In fact, other known TPO-catalysed reactions can also be catalysed by MPO, such as the coupling of di-iodotyrosine (DIT) to form thyroxine (T_4),[4] exchange of free iodide for covalently bound iodine in DIT (unpublished results), and the de-iodination of T_4.[5-8]

The dependence of leucocytes on iodine metabolism is illustrated by reports that they are capable of concentrating iodide,[9] and that their utilization of thyroid hormones is three to four times greater during phagocytosis than the resting thyroid hormone requirement.[7] Although MPO in the leucocyte catalyses both iodination and chlorination reactions, which are both effective during phagocytosis as part of the bactericidal actions of the inflammatory response, the iodination reaction is more effective in antimicrobial systems, so that iodination is favoured above the chlorination reaction.[7] Furthermore, during increased activity of leucocytes, as in acute infections, several changes in the thyroid gland and its peripheral hormone levels have been observed.[10]

The mechanism of action of non-steroidal anti-inflammatory drugs is limited to the finding that they block prostaglandin synthesis.[11] In the latter process, a peroxidase enzyme is also involved.[12] The classic prostaglandin synthesis inhibitor, indomethacin, has been shown to cause a dramatic decrease in circulating thyroid hormone levels.[13] On the other hand, thyroid status affects oxygen consumption and thyroxine de-iodination by human leucocytes.[5] These processes are involved in H_2O_2 production, on which MPO-catalysed de-iodination and other MPO-catalysed reactions are dependent.

In view of the multifunctional nature of the peroxidase enzymes and the striking functional similarities between TPO in the thyroid and MPO in the leucocyte, the usefulness of the leucocyte for studying the thyroid hormone metabolism[6] and the influence of non-steroidal anti-inflammatory drugs and antithyroid substances seems rational and important. As it was observed in the present study that the loss of cell integrity caused an increase in MPO-catalysed reactions of the leucocyte, sonicated normal human leucocyte suspensions were used as a model system for the studies on MPO.

MATERIALS AND METHODS

Preparation of Leucocytes

Human leucocytes were prepared mainly according to the method of Olsson *et al.*[14] Several batches of 450 ml

fresh heparinized whole blood from healthy normal individuals were obtained from the Western Province Blood Transfusion Service. Approximately 30 ml blood was placed in 15 × 50-mm siliconized glass tubes containing 10 ml of 4% dextran in 0,9% NaCl, and was carefully mixed to prevent haemolysis. The dextran facilitates sedimentation of erythrocytes, while the white blood cells remain in suspension. The mixture was allowed to stand at 4°C for 1 hour. The leucocyte-rich plasma was sucked off carefully with siliconized Pasteur pipettes and was centrifuged at 200 *g* for 10 minutes at 4°C.

The supernatant was pipetted off and the leucocyte pellet was resuspended in 20 ml 0,8% NH_4Cl per tube in order to lyse the contaminating red cells. The suspension was again centrifuged at 80 *g* for 10 minutes at 4°C. The pellet was resuspended in 20 ml balanced salt solution per tube and was centrifuged at 80 *g* for 10 minutes at 4°C. The relatively pure leucocytes were resuspended in 4 ml balanced salt solution.

Viability Test of Leucocytes and Sonication of Cells

Trypan blue 0,1% in 0,05M PO_4 buffer, pH 7,0, containing 1% glucose and 0,45% NaCl, was used to test the viability of leucocytes. Equal volumes of the white cell suspension and of the trypan blue solution were mixed on a haemocytometer slide, and the number of unstained cells per 100 total cells was counted under a light microscope. The number of unstained cells per 100 indicates the percentage viability, which was well above 90% for every preparation.

A total of 500 µl of the original cell suspension was sonicated in small test tubes for 15 seconds in ice, using an MSE 150-watt ultrasonic disintegrator, MK 2, with a 4-mm titanium probe and 12-µm peak-to-peak amplitude. Microscopical examination indicated total cell rupture under these conditions.

Balanced Salt Solution

The balanced salt solution contained 6,8 g NaCl, 0,125 g NaH_2PO_4, 0,4 g KCl, 2,2 g $NaHCO_3$, 0,2 g $CaCl_2$, 1,0 g glucose and 0,1 g $MgSO_4$ made up to 1 litre in de-ionized, all-glass distilled water.

^{131}I-DIT and ^{125}I-T_4

^{131}I-DIT was prepared from adult rat (200 g) thyroids 24 hours after injection of 300 µCi ^{131}I per rat. Ether-anaesthetized rats were bled to death and the thyroid was removed, dissected free of connective tissue and homogenized in 0,8 ml 0,1M KCl at 4°C. Thyroglobulin was prepared by sucrose density gradients (5 - 40%), dialysed against 0,067M PO_4 buffer containing 0,1M KCl, pH 7,01, and digested with pronase under reduced pressure at 37°C for 5 hours. The digest was chromatographed on Whatman 3 MM paper in butanol : acetic acid : water (BAW), the DIT band was identified by radio-autography, and was cut out and extracted with methanol : ammonia (3 : 1 v/v). The extract was concentrated to near dryness at 15°C in an Evapomix (Buchler Instruments) and rechromatographed in BAW. Fresh ^{131}I-DIT samples prepared in this way were more than 90% pure, as judged from radio-active counts on BAW chromatograms.

^{125}I-T_4 samples with specific activities between 40 and 60 µCi/µg T_4 were obtained from the Radiochemical Centre, Amersham.

Incubation System

Only incubation systems in which H_2O_2 was generated were used, and incubation time was started with the addition of glucose oxidase, and was terminated after 20 minutes at 37°C by direct application of 25-µl samples to the chromatography paper, unless otherwise stated.

For iodination experiments crystalline bovine serum albumin (BSA) was used at a final concentration of 500 µg/ml. The remainder of the components was as follows: 100 µl whole cell suspension or sonicated cell suspension representing approximately 4 × 10^5 white cells per reaction tube, ^{131}I-iodide (1 mM), glucose (10 mg/ml), glucose oxidase (0,04 U/ml) and phosphate buffer, pH 7,0 (0,067M). The total volume was 500 µl.

For ^{125}I-T_4 delabelling or iodine exchange in ^{131}I-DIT, 50 µM ^{125}I-T_4 or ^{131}I-DIT was used in the incubation system instead of BSA.

Chromatography and Counting of Samples

An aliquot (usually 25 µl) of the incubation mixture was applied along a 3-5-cm line, 2,5 cm from one end of a strip of Whatman No. 1 paper in duplicate. The paper was immediately put into the chromatography tank. For iodination experiments short-time chromatography (≈ 2 hours) was used, whereby sufficient time was allowed for the solvent front to move from the origin for a distance of about 10 cm; this was sufficient for the free iodide band to separate completely from the iodinated BSA, which remained at the origin.

When ^{131}I-DIT or ^{125}I-T_4 was used as substrate for iodine exchange or T_4 delabelling experiments, chromatography was continued overnight (≈ 16 hours). The papers were subsequently exposed to X-ray film for radio-autography, and the radioactive bands on the chromatogram were located by referring to the radio-autogram. These were cut out of the paper together with all the sections between the bands, and counted in a dual-channel Packard counter, accumulating sufficient counts to provide a standard deviation of less than ±3%. The fraction of the total ^{131}I and ^{125}I on the paper present in each section of the chromatogram was determined. When ^{125}I and ^{131}I activities were counted together, a correction was made for the ^{131}I spillover into the ^{125}I counts; this was approximately 14%.

Anti-inflammatory and Antithyroid Drugs

Stock solutions (0,05M) of indomethacin (MW 357,8), naproxen (MW 230) and flufenamic acid (MW 282) were prepared by dissolving the reagents in 1M tris buffer, pH 11,4. The required concentrations (10^{-7} - 10^{-2}M) were

obtained by dilution in 0,067M PO_4, pH 7,04. Similar solutions were made from 1M tris buffer to act as reagent controls.

Methylmercapto-imidazole (MMI) (MW 114,6) was dissolved in 0,067M PO_4 buffer, pH 7,04, to the required dilutions.

RESULTS

Effect of the Incubation Medium, Phagocytosis of Latex Particles and Cell Integrity on Leucocyte-Catalysed Iodination of BSA

In an experiment in which whole white cells were used for the iodination of BSA in 0,067M PO_4, pH 7,04, and were compared with the iodination in a medium of balanced salt solution of the same pH, less than 10% of the BSA was iodinated after 20 minutes in the phosphate medium compared with over 20% in the balanced salt solution. In neither of the two incubation media did the addition of latex particles (Dow-latex, 10% in 0,9% NaCl; particle size 0,357 μm; Serva Entwicklungslabor, Heidelberg) stimulate the degree of iodination, presumably since the leucocytes were already stimulated maximally by the presence of BSA. When the incubation was continued for 18 hours, BSA iodination in the phosphate medium did not increase to any appreciable extent, whereas it rose to $\approx$ 40% in the balanced salt solution. Again, the presence of latex particles had no influence on the degree of BSA iodination. Nevertheless, it was clear that the cells were more active in the balanced salt solution and that the BSA could be iodinated by whole cells, presumably after the protein had been phagocytosed.

Experiments were then performed in the balanced salt solution to test the influence of cell integrity on iodination. The results are illustrated in Fig. 1A, in which the rate of iodination of BSA in whole cells is compared with that of sonicated cells. It was evident that sonication of leucocytes facilitated the process of iodination, presumably by releasing MPO from the phagosomes in order to make free contact with the BSA, whereas the rate of iodination in whole cells is most likely a measure of the rate of phagocytosis. More than 70% of the BSA added to the medium was iodinated within 30 minutes of incubation by sonicated cells, whereas about 20% was iodinated by whole cells within the same period.

In order to establish the experimental conditions for MPO-catalysed BSA iodination, a time study on sonicated cells was performed at shorter time intervals (Fig. 1B); this showed that the maximum of nearly 80% BSA iodination was already achieved at 15 minutes, and that iodinations for the 30- and 60-minute incubation periods were somewhat lower than those for 15-minute samples. A standard incubation time of 20 minutes was chosen for subsequent experiments.

Influence of Non-steroidal Anti-inflammatory Drugs (Indomethacin, Flufenamic Acid and Naproxen) on BSA Iodination

As the non-steroidal anti-inflammatory drugs are sparingly soluble in water, they were dissolved in 1M tris buffer and diluted to the required concentrations in

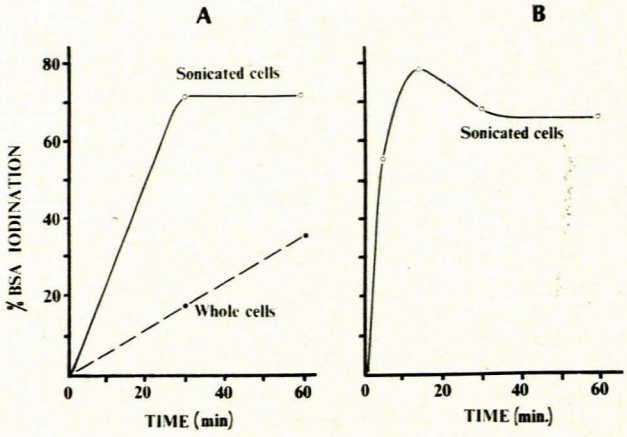

Fig. 1. Rate of BSA iodination by normal human whole leucocytes and sonicated cells measured at 30-minute intervals (A) and by sonicated cells in the same preparation at 5-, 10-, 15-, 30-, and 60-minute intervals (B). The incubation medium consisted of 500 μg/ml BSA, 100 μl whole cells or 100 μl sonicated cell suspension ($\approx 4 \times 10^5$ leucocytes), 1 mM ^{131}I-iodide, 10 mg/ml glucose and 0,04 U/ml glucose oxidase in balanced salt solution to a total volume of 500 μl. Incubation time was started with the addition of glucose oxidase. At required intervals 25-μl samples were placed on Whatman No. 1 filter paper and chromatographed in BAW for 2 hours. The radioactive bands were identified by radio-autography for separate counts of iodinated BSA (band remaining at the origin) and ^{131}I-iodide band (which moved close to the solvent front).

0,067M PO_4 buffer, pH 7,04. Experiments were performed on equivalent tris concentrations to establish whether they had any influence on BSA iodination of sonicated leucocytes, but no concentration response was observed. Nevertheless, for every drug concentration a control experiment was performed with the same amount of drug solvent. The percentage BSA iodination of the control was taken as 100%, and inhibition caused by a particular drug concentration was calculated accordingly.

For every experiment a fresh batch of white cells was prepared from blood drawn freshly on the same day. The preparation was completed in less than 6 hours, after which time the incubation experiment was performed immediately. The fact that different degrees of iodination were obtained in different control experiments could be ascribed to the variation in white cell concentrations of the various blood samples used from day to day. No attempt was made to match the white cell counts of different preparations for each set of experiments.

Fig. 2A, B and C summarizes the results obtained with indomethacin (10^{-7} - 10^{-3}M), flufenamic acid (10^{-7} - 10^{-3}M) and naproxen (10^{-7} - 10^{-2}M) respectively. Indomethacin showed little inhibitory effect on MPO-catalysed BSA iodination of sonicated leucocytes, except at concentrations of 100 μM and 1 mM, which caused 12% and 15% inhibition respectively. Flufenamic acid showed greater inhibitory effects of approximately 20 - 24% between 10 μM and 1 mM concentrations, whereas naproxen, with 11% at a 1-mM concentration, was the least effective inhibitor.

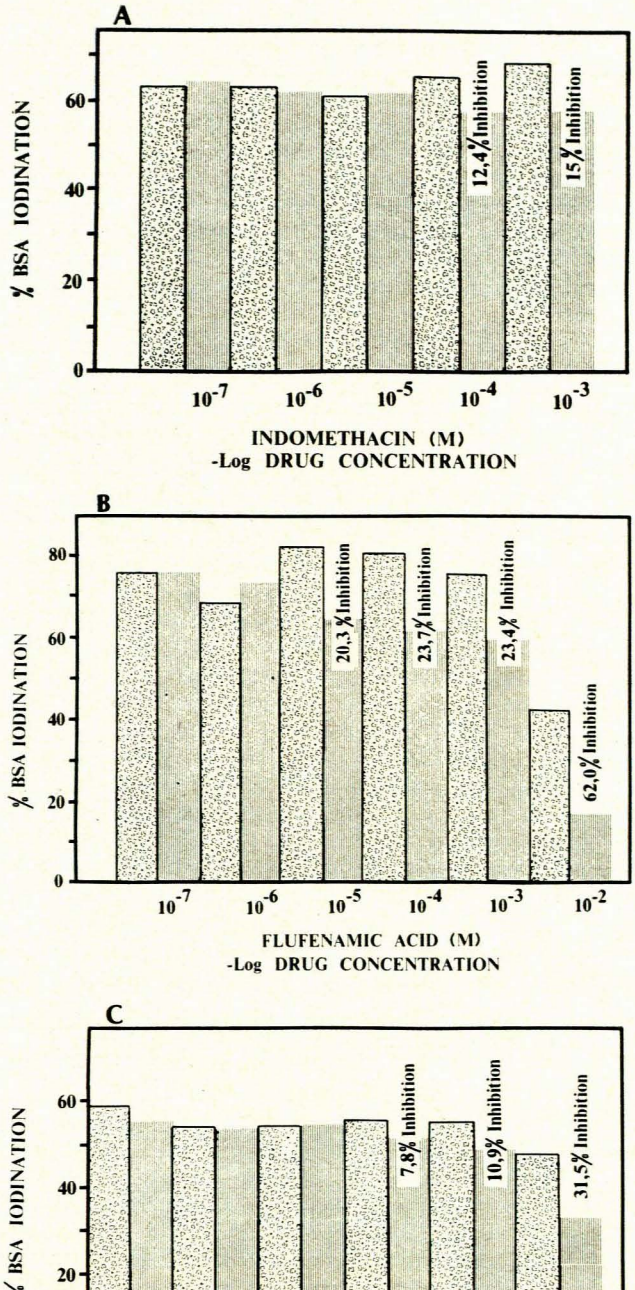

Fig. 2. The influence of indomethacin (10^{-7} - 10^{-3}M) (A), flufenamic acid (10^{-7} - 10^{-3}M) (B), and naproxen (10^{-7} - 10^{-2}M) (C) on BSA iodination of freshly prepared sonicated normal human leucocytes. The patterned histograms represent percentage control BSA iodination, and the hatched blocks represent the percentage BSA iodination of experiments containing the drug concentrations as indicated. Other components and methods were the same as indicated in the legend to Fig. 1.

Influence of the Antithyroid Substance MMI on MPO-Catalysed Reactions

The first object of these experiments was to see whether MPO contained in sonicated leucocyte preparations was able to catalyse other activities known to be catalysed by thyroid peroxidase as well, such as iodine exchange in DIT and de-iodination of T_4, apart from being able to facilitate BSA iodination. The results shown in Fig. 3 indicated that all these reactions were indeed catalysed by MPO of sonicated leucocytes.

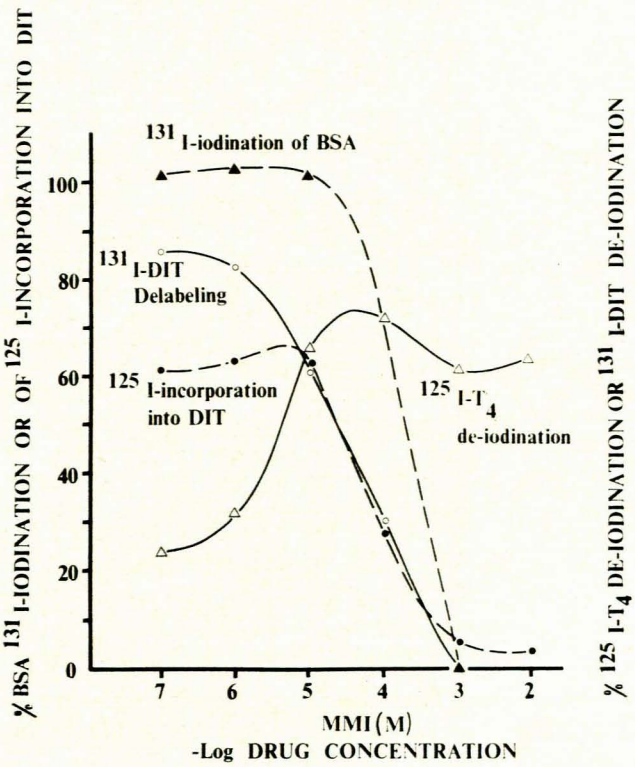

Fig. 3. The influence of MMI on BSA iodination (▲---▲), ^{125}I-iodide exchange in ^{131}I-DIT (●---●) representing ^{125}I incorporation into ^{131}I-DIT, and (○——○) representing the ^{131}I delabelling of ^{131}I-DIT, and on the de-iodination of ^{125}I-T_4 (△——△) representing ^{125}I delabelling of ^{125}I-T_4. The components and methods were the same as indicated in the legend to Fig. 1, except that the incubation medium for the iodine exchange experiments contained 50 μM ^{131}I-DIT, whereas for the de-iodination experiments on T_4 it contained 50 μM ^{125}I-T_4 instead of BSA. For BSA iodination, ^{131}I-DIT de-iodination and ^{125}I incorporation into ^{131}I-DIT the control values (i.e. the mean value obtained without MMI) were taken as 100%, and experimental values were calculated in terms of the control. For ^{125}I-T_4 de-iodination the percentage stimulation caused by MMI is the percentage over and above that of the control value.

Workers have shown that the antithyroid drug MMI inhibited TPO-catalysed reactions such as iodination,[15] iodine exchange in ^{131}I-DIT[16] and delabelling of ^{131}I-T_4,[16] but that MPO-catalysed delabelling of ^{125}I-T_4 was stimulated by MMI, while the same drug inhibited the latter re-

action when MPO was replaced by lactoperoxidase or horseradish peroxidase.[7] It was therefore decided to test the antithyroid substance MMI at various concentrations on the different reactions catalysed by MPO of sonicated leucocytes in a H_2O_2-generating system.

On BSA iodination MMI concentrations between 100 nM and 100 μM showed a slight but consistent stimulating influence above those of control values. Concentrations of MMI greater than 100 μM caused a marked inhibition, so that iodination was completely suppressed at 1 mM.

MPO-catalysed iodine exchange in ^{131}I-DIT indicated that ^{125}I incorporation into ^{131}I-DIT remained at a 60% level of that of the control between 10^{-7} and 10^{-5}M of MMI (i.e. inhibited by about 40%), whereas the ^{131}I-DIT delabelling process was inhibited by only 10 - 15% at the same MMI concentrations. At higher MMI concentrations (10^{-5} - 10^{-3}M) a linear loss of ^{131}I label occurred, as well as a loss of the ability of MPO to affect ^{125}I incorporation into ^{131}I-DIT.

Unlike MPO-catalysed delabelling of ^{131}I-DIT, which was inhibited by all concentrations of MMI, ^{125}I-T_4 delabelling was stimulated at all levels of MMI (10^{-7} to 10^{-2}M) to a remarkable extent.

DISCUSSION

The resting metabolism of polymorphonuclear lymphocytes is based largely on anaerobic glycolysis. However, during phagocytosis, which can be experimentally induced with latex particles, the cells display an increased oxygen consumption[17] and increased thyroid hormone utilization.[6] These processes are associated with an increased bactericidal effect which involves iodination processes, H_2O_2 production and an increased superoxide anion (O^-_2) production.[18] This superoxide radical with its superior reactivity is suggestive of a possible killing agent in leucocytes. Yet at the same time it is a dangerous substance in inflammatory conditions and therefore needs to be inactivated by superoxide dismutase with formation of H_2O_2. Nevertheless, it has been implied that the circumstances under which O^-_2 is produced are suggestive of both O^-_2 as well as H_2O_2 participating in bacterial killing.[18] At the same time the O^-_2 may be responsible for the iodination process catalysed by the H_2O_2-dependent MPO of the leucocyte.

Preliminary experiments indicated that leucocyte phagocytosis during inflammatory conditions is suppressed by anti-inflammatory non-steroidal agents because of inhibition of H_2O_2 generation and of O^-_2 formation.[19] Our studies on TPO[20] have shown that the anti-inflammatory substances inhibited iodination catalysed by several peroxidases of plant and animal origin. Furthermore, these drugs also inhibited H_2O_2 generation on which the peroxidase activity is dependent. In fact, a drug like naproxen had little if any effect on the peroxidase enzyme, but inhibited almost exclusively *in vitro* H_2O_2 generation at concentrations equivalent to calculated blood levels expected from therapeutic dose regimens.

If iodination is favoured above chlorination as a process of inactivating invading organisms by MPO of leucocytes and macrophages,[7] it is fortuitous that the non-steroidal anti-inflammatory reagents tested seem to be less effective inhibitors of MPO-catalysed iodinations than of TPO-catalysed iodinations. It should, however, be kept in mind that the *in vitro* experimental results reported in the present study were not obtained with purified MPO but on sonicated leucocytes, whereas the observations on TPO-catalysed iodinations were made on two different purified enzymes. Nevertheless, on the basis of our present data it would appear as if therapeutic administration of non-steroidal anti-inflammatory agents after long-term treatment may interfere with the physiological role of TPO in thyroid function rather than with MPO-catalysed iodination in the inflammatory response.

Whether leucocytes make use of all the MPO-catalysed reactions, as can be demonstrated by *in vitro* studies and as indicated by the present data, is a matter of speculation. Nevertheless, it is interesting that, as far as can be ascertained, MPO has been shown to facilitate all H_2O_2-dependent TPO-catalysed reactions such as oxidation of iodide, iodination of tyrosyl residues, coupling of DIT to form T_4, and exchange of inorganic iodide for organic iodine in DIT. Another TPO-catalysed reaction facilitated by MPO is the de-iodination of T_4. With TPO and horseradish peroxidase-catalysed iodination of thyroglobulin, conformational changes of thyroglobulin had been observed.[21,22] It is still unknown whether the latter reaction can also be catalysed by MPO. Apart from these reactions, the leucocyte can also concentrate iodide.[9] It seems likely that the rationale for some of these reactions could be sought in the common goal of providing and utilizing inorganic and organic iodine in extracellular fluid for bactericidal processes.

The antithyroid drug MMI inhibits the de-iodination of DIT (as part of the exchange phenomenon), but stimulates the de-iodination of T_4 at all concentrations of MMI. Stimulation of thyroxine de-iodination by MMI at a single and final concentration of 1 mM has been demonstrated before[7] and is confirmed by the present study. On the other hand, MMI is a well-known and powerful inhibitor of all known TPO-catalysed reactions, including T_4 de-iodination. Although these opposing effects of MMI are at present difficult to understand in two enzymes (i.e. TPO and MPO), which are both able to catalyse the same reactions, it would appear as if MMI could be an ideal anti-inflammatory drug if its effects on the thyroid could be prevented. Its differential influence on ^{125}I incorporation into DIT as compared with delabelling of ^{131}I in DIT in the exchange phenomenon catalysed by sonicated neutrophils at MMI concentrations up to 10^{-5}M, adds to the problem of understanding the mechanism of action of MMI.

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Popliteal False Aneurysm Complicating Osteochondroma

A Case Report

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SUMMARY

A false aneurysm of the popliteal artery, caused by an exostosis in a patient with multiple exostoses, is described.

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CASE REPORT

A 17-year-old Black youth presented at the Orthopaedic Clinic with a 2-week history of a painful, rapidly progressive swelling on the anteromedial aspect of the lower left thigh. There was no history of trauma. On examination a popliteal aneurysm with an audible bruit was found. The patient was also noted to have prominent exostoses, principally around the knee joints and, to a lesser extent, around the shoulder and wrist joints. Of 8 sibs, 3 are similarly affected.

Radiological Skeletal Survey

Multiple osteocartilaginous exostoses were demonstrated around all the larger joints. The pelvis and scapulas were also involved. There were no spinal exostoses. Radiographs of the left knee demonstrated abnormal modelling of the distal femur and of the proximal tibia and fibula. A well-defined mass of soft tissue density surrounded the offending exostosis (Fig. 1).

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Left Femoral Angiography

This revealed a false aneurysm of the popliteal artery (Fig. 2) which was explored by a surgeon attached to the vascular unit of King Edward VIII Hospital. A large false aneurysm deep to the adductor muscles, arising from a lateral defect in the upper popliteal artery, was found, the defect having been caused by the large exostosis arising immediately proximal to the femoral epiphysis. The exostosis was removed, the defect in the popliteal artery was sutured, and the postoperative course was uneventful.

Histology

On microscopical examination the appearance of the specimen was consistent with a typical exostosis, the cartilage cap having been replaced by lamellar bone.

DISCUSSION

Diaphyseal aclasis is a bone dysplasia affecting long and flat bones, the former more frequently. The disorder, as in this patient, tends to be familial, and is then usually symmetrical. Inheritance is due to an autosomal dominant gene. Isolated exostoses may occur on a sporadic basis and do not tend to be familial.

Complications due to the exostoses arise mainly through compression of blood vessels, tendons, nerves and spinal cord. Other complications include bursa formation, stress fractures, modelling deformities and secondary degenerative changes in the hip joints. Malignant change is uncommon, and the incidence of osteochondrosarcoma is less than 5%, occurring more commonly in the flat bones.

Because the knee is frequently involved in diaphyseal aclasis, the popliteal artery is particularly vulnerable to pressure effects and injury. When this happens it is