REDUCED EXUDATION AND INCREASED TISSUE PROLIFERATION DURING CHRONIC INFLAMMATION IN RATS DEPRIVED OF ENDOGENOUS PROSTAGLANDIN PRECURSORS

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ABSTRACT

Two models of chronic inflammation were studied in rats deprived of endogenous precursors of prostaglandins by feeding the animals on essential fatty acid deficient (EFAD) food. During kaolin-induced pouch-granuloma, exudate production was markedly reduced in EFAD rats, when compared with normal animals. The exudates from normal rats contained large amounts of PGE, but in the exudates from EFAD rats the amount of PGE was very markedly reduced. Similarly, with carrageenan-impregnated polyether sponges, the exudative component of inflammation was reduced in EFAD rats. However, the proliferative component was significantly increased, particularly in relation to the stunted growth of EFAD rats. Sponge exudates from EFAD rats contained fewer leucocytes than those from normal animals but the fall in leucocyte count was much smaller than the very marked reduction in PGE activity. EFAD rats also exhibited a significant increase in adrenal weights.

The results are discussed in the light of the ambivalent (pro- or anti-inflammatory) role of endogenous PGs. It appears that, in the proliferative phase of inflammation, the anti-inflammatory role of PGs is more dominant.

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INTRODUCTION

Feeding mammals with an essential fatty acid deficient (EFAD) diet lacking linoleic acid (18:2, n-6) results in a marked reduction in tissue levels of bishomo-γ-linoleic acid (20:3, n-6) and arachidonic acid (20:4, n-6). In EFAD rats, the arachidonic acid (AA) content of membrane phospholipids is replaced by eicosatrienoic acid (20:3, n-9), which is not a substrate (1), but rather a competitive inhibitor of the cyclo-oxygenase step in the biosynthesis of prostaglandins (PGs) (2). Such deficient animals exhibit a reduced oedema, following the injection of carrageenan into the hind paw (3), but the response can readily be restored to the level of normal rats by injecting AA with the carrageenan (4). In normal rats the local administration of arachidonic acid, but not eicosatrienoic acid (20:3, n-9), results in potentiation of carrageenan-induced hind paw oedema (4,5). In contrast, local administration of BPP_{9a}, a bradykinin potentiating peptide (6), enhanced the poorly developed carrageenan oedema of EFAD rats to the same extent as it did in normal rats (4). Denko (7) has also shown that acute swelling, induced by urate crystal injection into the hind paw of rats, is markedly reduced in EFAD animals. This reduction was reversed by concomitant injection of very low doses of PGE_1, PGE_2, PGA_2 or $PGF_{2\alpha}$. Thus, in the absence of endogenous PG-precursors, there is a selective abolition of the, so called, PG-component of acute inflammation, while the non-PG-mediated component remains unaffected.

That the reduced inflammatory response in EFAD rats is due to lack of PGs and their intermediates is further indicated by the fact that platelets from these animals do not release prostaglandin (PG)-endoperoxides, thromboxane A_2 or PGE when aggregated with collagen *in vitro* (8). The release of these substances was restored following addition of AA to incubation mixture (8) showing that, in EFAD animals, the impaired release of PGs was due to a shortage of endogenous precursors and not to a malfunction of the cyclo-oxygenase.

The results obtained with acute inflammation, in rats deprived of endogenous PG-precursors, are in agreement with the view that PGs are pro-inflammatory, through potentiation of the actions of other inflammatory mediators such as bradykinin (9). Furthermore, the initial results obtained with EFAD rats, as discussed above, suggested that such animals might be a useful model in discriminating between PG and non-PG mediated components of inflammation.

In order to investigate the importance of endogenous PGs in more chronic inflammatory situations than the rat paw oedema, we have studied the production of pouch-granuloma and the foreign body-granuloma in normal and EFAD rats. It will be shown that, in the absence of endogenous PGs, not all components of inflammation are affected in the same way.

MATERIALS AND METHODS

Male albino rats of an inbred Wistar strain (Animal Farm TNO, Zeist) weighing 185-210 g were used throughout the investigation. Deprivation of endogenous PG precursors was achieved by maintaining the animals on an EFAD diet as described previously (3). Control rats were fed on a diet, 3.5% of the calorific value of which consisted of linoleic acid. The rats raised on EFAD food showed retarded growth (25-35% at the time of the experiment), ruffled hair and occasionally scaly tails, which are well known symptoms of the EFAD condition. Fatty acid analysis of erythrocytes had shown earlier that, whereas in normal rats the ratio of eicosatrienoic acid/arachidonic acid was 0.013, in our EFAD rats a ratio of 1.97 was found (8). A ratio greater than 0.4 is considered as a criterion of essential fatty acid deficiency (10).

In two series of experiments (pouch granuloma and sponge granuloma) the rats were caged in Makrolons containing sawdust. However, it was found that the animals were chewing the sawdust and the EFAD rats suddenly showed a somewhat higher growth rate than usually observed with these animals. Therefore, in a third experiment (the second sponge granuloma experiment), the sawdust bedding was replaced with Sol "Speedi-Dry" (Metallochemie, Ramondt, Holland).

For induction of the pouch-granuloma essentially the same technique as that of Selye (11) was used. The hair on the back of the rats was shaved off and the animals lightly anaesthetised with ether. 6 ml of air were injected, subcutaneously, in the dorso-lumbar region and with the needle in situ, 4 ml of a sterilised 10% kaolin suspension in water were injected into the air pouch. On day 4 or 8 (specified in the table) the rats were killed with chloroform, the skin over the pouch was cut, the exudate was withdrawn from the pouch with a syringe and the volume recorded. The PG content of 4 control and 2 EFAD exudates was determined on day 4 as described below.

For induction of foreign body granuloma, the sponge implantation technique, similar to that described recently by other authors (12,13), was used in an initial experiment, with slight modification in a second experiment. Polyether sponges (1.2 x 1.2 x 0.5 cm foam sheet) were soaked in 2% carrageenan solution (Viscarin, carrageenan Na, Marine Colloids In., Springfield, N.J.) for 1 minute, dried for 24 hours at room temperature (first exp.) or 37°C (second exp.) and weighed. Two sponges were implanted subcutaneously, under light ether anaesthesia, into the backs of each rat (one sponge on each side) and the incision closed by stitching. 8 Days after the implantation the rats were killed with chloroform and the sponges, with the surrounding connective tissue, were removed.

In the second experiment, fluid beneath the outer connective tissue capsule was removed with a syringe before cutting the granuloma and sponge out of the rat. In this experiment, the capsule was also removed and the fluid in the sponge squeezed out. The total volume of both fluids was then measured. The PG content of the fluid withdrawn from beneath the capsule and the leucocyte count of the sponge fluid were determined as described below. The total wet weights of the granulomata were measured in both experiments and thereafter they were dried for 24 hours at 80°C and reweighed. The sponge weights before implantation were subtracted from the values obtained after removal and drying of the granulomata and expressed as Δdry weight. The dry weights of the sponges plus granulomata were subtracted from the total wet weights (without the free exudation fluid in the second experiment) to give an indication of the amount of exudation. The adrenals were also removed, together with the granulomata and both adrenals from each rat weighed together. Body weight of the animals was recorded on the day of implantation and on the day of autopsy. By careful allocation of animals to each group, similar initial mean weights were obtained for each group (controls and EFAD). Since, in the second experiment, body weight changes, over the 8 day period of the experiment, were different in EFAD and control rats, granuloma formation was expressed as a proliferation index, defined as Δdry weight of the sponges (mg)/Δbody weight.

For measurement of PG content the pouch-granuloma exudates were passed through an Amberlite XAD-2 column to which indomethacin (400 μg/ml) was added to prevent further PG synthesis by cells on the column. The lipids absorbed to the resin were recovered by washing with methanol. The extract was evaporated to dryness *in vacuo* in a rotatory evaporator and resuspended in 5 ml chloroform. This extract was stored in a refrigerator until required for bioassay on the rat stomach strip (14) superfused with Krebs containing a mixture of antagonists (15) and indomethacin (1 μg/ml). PGE_2 was used as a standard and the extracts were assayed using a three point bracketing design.

For measurement of PG-like material in sponge granuloma fluid (second experiment), fluids removed from the capsules of both granulomata in each rat were pooled and centrifuged at 2,500 rpm for 5 min. The supernatant was removed and assayed simultaneously on the rat stomach strip and rat colon, superfused at 0.4 ml/min with Krebs solution containing antagonists (15) and indomethacin (2 μg/ml), against PGE_2 and $PGF_{2\alpha}$, using a modification (16) of the mineral oil technique of Ferreira & de Souza Costa (17). As little or no rat colon - contracting activity (<200 pg $PGF_{2\alpha}$) was found, the PG-like activity was expressed as PGE. The detection limit of the assay was 200 pg PGE_2.

In order to obtain some additional information on exudation, total leucocytes in the pooled fluid squeezed out

of both sponges from each rat (second experiment) were counted in a Coulter Counter, using 40 µl aliquots, after lysing eythrocytes with zaponin (Coulter Electronics Ltd., Harpenden, England).

RESULTS

The results obtained on the pouch-granuloma model are shown in Table 1. It can be seen that in EFAD rats there was a very marked suppression of exudate production when compared with normal rats. The difference was more pronounced with the day 4 exudate than with that measured on day 8. Large amounts of PGE-like material were present in the day 4 exudates from normal rats, but in the exudate from EFAD rats the amount of PGE was considerably reduced. Although there was insufficient fluid in EFAD animals for more than 2 PG bioassays, the lowest control value (22 ng/ml) was much higher than the highest EFAD value (10.7 ng/ml) in the exudates which were large enough for PG assay.

Table I. Effect of EFAD on production of exudate and PG-like material in kaolin-induced pouch granuloma.

	Normal	EFAD	p<
Exudate (ml):			
day 4	1.38±0.17(8)	0.25±0.02(8)	0.0005
day 8	4.59±0.88(8)	1.94±0.54(8)	0.012
PG-like material (PGE_2 equiv. ng/ml)	129 ±49 (4)	6.2(1.35-10.70)(2)	

Mean values are given ± s.e.m. The range of values is given for PG-like material in EFAD rats. Numbers of observations are given in brackets. PG-like material was estimated in day 4 exudates. Significance (EFAD v. Normal) was determined by one-tailed Student's t-test.

The results of the first experiment with implanted sponges are shown in Table 2. In this experiment, the rats were caged in Makrolons with sawdust bedding. The increase in the body weight of the EFAD rats over 8 days was not significantly different from that of normal rats over the same time period. The weight of the fluid contained by the gra-

nuloma of EFAD rats, though slightly reduced, was not significantly different from controls. In contrast, there was a marked increase in the dry weight of the granulomata from the EFAD rats. The increase was even more pronounced when judged on the basis of the proliferation index, in which the weight of the granulation is related to the increase in body weight over the period of the experiment. It was also found that the adrenals in the EFAD rats were considerably larger than those of normal animals.

Table II. Effect of EFAD on production of granuloma induced by carrageenan sponge implantation (exp.1).

	Normal	EFAD	p<
Initial body wt (g)	188.3±1.1 (6)	186.0±1.9 (6)	N.S.
Δ Body wt (g)	19.1±1.6 (6)	18.4±1.6 (6)	N.S.
Granuloma: Δ Dry wt (mg)	101 ±23 (12)	184 ±39 (12)	0.05
Total fluid (mg) (wet wt-dry wt)	1106 ±96 (12)	1039 ±84 (12)	N.S.
Proliferation index (ΔDry wt/ΔBody wt)	5.2 ±1.2(12)	10.1±2.1(12)	0.05
Adrenal wt(mg/100g)	13.7 ±0.95(6)	17.5±1.3 (6)	0.05

Mean values are given ± s.e.m. Numbers of observations are given in brackets. Significance (EFAD v. Normal) was determined by one-tailed Student's t-test (N.S.=non significant).

The results of the second experiment with implanted sponges are shown in Tables 3 and 4. In contrast to the EFAD rats in the initial experiment, which were caged with sawdust, EFAD rats caged with Speedi-Dri grit bedding showed a significantly smaller increase in body weight when compared with controls (Table 3). Furthermore, whereas the dry granuloma weight increased in EFAD rats caged with sawdust (Table 2), those caged with grit exhibited a significantly decreased granuloma formation. However, when expressing the granuloma weight in terms of the increase in body weight (proliferation index), granuloma formation was significantly increased when compared with controls (Table 3)

Table III. Effect of EFAD on production of granuloma induced by carrageenan sponge implantation (experiment 2).

	Normal	EFAD	p<
Initial body wt (g)	189.8±1.2 (5)	191.6±2.4 (5)	N.S.
Δ Body wt (g)	20.2±2.3 (5)	6.2±1.8 (5)	0.01
Granuloma: Δ Dry wt (mg)	379.4±33.6(10)	240.5±25.2(9)†	0.01
Total fluid (mg) (wet wt-dry wt)	2502 ±146 (10)	1854 ±55 (9)†	0.001
Proliferation index (ΔDry wt/ΔBody wt)	19.8±2.9 (10)	53.5±11.9(9)†	0.025
Adrenal wt(mg/100g)	32.2±1.3 (5)	35.8±0.7 (5)	0.01

Mean values are given ± s.e.m. Numbers of observations are given in brackets (†one sponge from EFAD rats was not dry after 24h at 80°C and this value has been excluded). Significance (EFAD v. Normal) was determined by one-tailed Mann-Whitney U test (N.S.=non significant).

Table IV. Effect of EFAD on various parameters of exudation induced by carrageenan sponge implantation (experiment 2).

	Normal	EFAD	p<
Exudate vol. (ml)	1.95±0.15(5)	1.35±0.09(5)	0.01
Leucocyte count (10^6 cells/ml)	12.0±3.1 (5)	5.5 ±0.6 (5)	0.05
PG-like material (PGE_2 equiv. ng/ml)	32.8±5.1 (5)	4.0 ±4.0 (4)†	0.01

Values are means ± s.e.m. from both sponges per rat. Numbers of observations are given in brackets (†only one of 4 measurable EFAD exudates contained detectable amounts of PG-like material). Values for exudate volume represent total, capsular + sponge, fluid; those for leucocyte count were obtained from sponge fluid and those for PG-like material from capsular fluid. Significance (EFAD v. Normal) was determined by one tailed Mann-Whitney U Test.

thus confirming the initial results. Total fluid content of the granulomata from EFAD rats was significantly reduced, when compared with normal rats (Table 3), as was the amount of free exudate removed from the capsule space and sponges (Table 4). Adrenal weights were also increased in the EFAD rats, confirming the results from the first experiment.

Analysis of the constituents of the exudate removed from the sponges (Table 4) revealed a significant reduction in the leucocyte count in exudates from EFAD rats. PG-like material was only present in detectable amounts in one of 4 EFAD exudates. There was an insufficient amount of exudate in the fifth EFAD rat for PG assay.

DISCUSSION

The results of the present experiments indicate that, in rats which are deficient in precursors of PGs, the exudative phases of kaolin- and carrageenan-induced granulomata are reduced, whereas tissue proliferation is increased. Although exudation was significantly reduced by EFAD in kaolin-pouch inflammation, exudation was only significantly reduced in one of the two sponge experiments. In two subsequent (unpublished) experiments, utilizing EFAD animals, exudation was also only significantly reduced in one experiment. Sponge implantation is not the best model to study exudation, since there is a limit to the absorbance of the sponges and granuloma formation further affects the absorbance. Moreover, exudation is a characteristic of acute inflammation and since inflammation after sponge implantation was determined on day 8 (semi-chronic), it is likely that exudation was altered by other mechanisms than those involved in acute exudation. However, the trend in EFAD animals was always towards a reduction in exudation, as shown in the kaolin-pouch experiments. This reduced exudation in EFAD rats is in agreement with earlier results, showing that acute, hind-paw oedema, induced by carrageenan or kaolin, is reduced by EFAD (3,4). Since PGE-like material was also markedly reduced in exudates from both types of inflammation and since PGE induces oedema in the rat (18), it seems likely that the effects on exudation and PGE production are causally related. The very small amounts of PG in EFAD exudates support earlier results showing that platelets from EFAD rats release exceedingly small amounts of PGs, PG endoperoxides and thromboxane A_2 (8).

The levels of PGE detected in kaolin-induced exudates in normal rats are similar to those found by Willis (19) in carrageenan-induced hind paw oedema, using parallel bioassay. However, Ohuchi et al. (20), using radioimmunoassay, detected less than 10 ng/ml PGE in exudates of carrageenan pouch granulomata. Furthermore, Lewis et al. (21) have recently found maximum levels of PG-like activity of only 30-40 ng/ml in kaolin-pouch exudates. These levels,

though, were detected in 6h exudates, whereas the present study involved assay of PG-like material in 96h exudates. It is probable that amounts of PGs detected in inflammatory models may be dependent on the mode of administration or the irritant and the period of inflammation, as well as on the method of assay.

The reduction in PGE activity in carrageenan sponge exudates of EFAD rats was parallelled by a reduction in leucocyte count. However, the reduction in PGE activity was much greater than the corresponding fall in leucocytes, which were still present in large numbers. The evidence indicating a role for PGE as an important chemotactic factor in the development of inflammatory exudation, has been briefly reviewed recently by Walker et al. (22). However, these authors found that, although fresh solutions of PGE_1 are chemotactic to rabbit peritoneal polymorphonuclear leucocytes (PMNLs) in vitro, they have no activity against rat peritoneal PMNLs or against such cells obtained from the blood of rabbits, rats or man. Furthermore, Shibuya et al. (23) have provided evidence suggesting that PGs might induce random movements of rabbit peritoneal PMNLs rather than initiating directional chemotactic migration. It should be noted that $PGF_{2\alpha}$ was the main PG used in their investigation, whereas Walker et al. (22) found significant, directed migration-inducing activity with PGE_1. That a marked reduction in PG production in EFAD rats only produces a relatively small reduction in leucocyte migration, as shown in our experiments, supports the suggestion that PGs do not play a major role in leucocyte migration in vivo. The fact that leucocytes retained by the sponges after squeezing were not determined in our experiments, as in other recent studies (12,13), does not alter this conclusion, although the discrepancy between the absolute numbers of migrating leucocytes in control and EFAD animals may be somewhat different from the value which we obtained. Vincent et al. (24) have shown that EFAD rats are leucopenic when compared with controls. Thus, in our experiments, the ratio of migrating leucocytes/total circulating leucocytes in EFAD animals was probably not much different from controls. A more rigorous study of leucocyte migration in EFAD granuloma appears to be warranted.

When calculated on the basis of the increase in body weight, the weight of the carrageenan granulation tissue was found to increase in EFAD rats, suggesting that PGs might inhibit granuloma formation in this model. In fact, PGE_2, injected into sub-cutaneous cotton pellets, has been shown to inhibit granuloma formation in rats (25). Also, PGE_2 and to a lesser extent PGE_1, have been shown to cause a delayed inhibition of the synthesis of bone collagen in vitro (26). Furthermore, low doses of indomethacin, an inhibitor of PG synthesis (9), have been shown to stimulate collagen synthesis in sponge granulomata after implantion for 7-21 days (27). However, the opposite effect was

found on connective tissue activation in human synovial tissue cultures, where PGEs stimulated hyaluronic acid synthesis (28). A stimulatory effect of PGs on labelled proline incorporation into various non-inflamed tissues has recently been suggested by Denko (29) on the basis of chronic experiments with EFAD rats. However, these rats were only fed on EFAD diet from weaning, instead of feeding the pregnant mothers; the tissue essential fatty acid content was not determined; and corn oil was administered at various intervals to keep the animals alive. It is also possible that collagen synthesis in inflamed tissue may differ in its sensitivity to PGs from that in normal tissue. We are, at present, investigating possible effects of PGs and EFAD on collagen synthesis and degradation during the production of carrageenan sponge-granuloma.

In the first sponge experiment the body weight gain over the 8 days of the experiment was similar for both control and EFAD animals, whereas in the second experiment the EFAD rats gained much less weight than the controls. This is almost certainly attributable to the different types of cage bedding used, since in two subsequent (unpublished) experiments, using EFAD rats caged with grit, the EFAD rats gained much less weight than the controls. The nature of the "growth-promoting factor" contained in the sawdust, which was eaten by the EFAD rats in the first experiment, is not known. It is unlikely to have been a precursor of endogenous PGs, because EFAD rats, which were kept on sawdust, still exhibited very markedly reduced amounts of PGs in the exudates. The factor may have inhibited the metabolism of EFAD rats, which is known to be higher than in normal animals (30). Since the metabolism of EFAD animals is different from that of normal animals, it seems likely that certain parameters of granuloma formation would also have been affected. In their studies on the effects of PGs on granuloma formation, DiPasquale et al. (25) expressed granuloma weight in terms of 100 g body weight, thus taking into account different weight changes between treatment groups. However, as the initial mean body weights of control and EFAD animals were similar in each of our sponge experiments, the more sensitive "proliferation index" was used, which relates granuloma formation to the increase in body weight over the same period. Using this index, granuloma formation was shown to be greater in EFAD rats than in controls in both sponge experiments. The importance of a correct index of proliferation is indicated by the fact that the Δdry weight increased in the first experiment but decreased in the second. In two subsequent experiments (unpublished), utilizing EFAD rats kept on Sol-Speedi Dri, Δdry weight also decreased. However, in all the experiments, the proliferation index was increased by EFAD. This further supports our contention that, in the first sponge experiment the "growth promoting factor" contained in the sawdust, also affected the growth of the granuloma and that this "interference" was overcome by the use of

the "proliferation index".

The overall effect of EFAD on the inflammatory models studied, namely decreased exudation and increased proliferation, underlines the current debate over the role of PGs as pro- or anti-inflammatory agents. The effects of PGEs on acute inflammatory oedemas in the rat appear to depend on the route of administration. Thus, injection of PGE_1 or PGE_2 into the hind paw, with carrageenin, results in potentiation of the swelling (31). Sub-cutaneous injection of pharmacological amounts of PGE_1 and PGE_2, on the other hand, inhibits carrageenin-induced hind paw oedema (32).

The reason for this discrepancy is unclear, but it may reflect a systemic anti-inflammatory action of PGE as opposed to a local pro-inflammatory action. No firm evidence appears to be available on the possible involvement of systemic inflammatory processes in either of the two models which we have studied. Thus, the differential effects of EFAD found in our investigation may indicate the local removal of the exudative (acute) and proliferation - inhibiting (chronic) effects of PGEs, representing the pro- and anti-inflammatory effects of these compounds respectively. Another explanation is that in the acute phase of inflammation PGs potentiate the inflammatory effects of other mediators (9). However, the participation of these other mediators (e.g. bradykinin, histamine) in the chronic phase of inflammation is unclear and may differ from their acute effects.

Apart from the effects of EFAD on the inflammatory response, the adrenal weights of EFAD rats, submitted to inflammatory stress, were significantly higher than in the normal controls. Since the proliferative component of carrageenan inflammation is also increased in EFAD, it is possible that the increased chronic phase of the inflammation, which this component represents, may result in greater stress, thus accounting for the larger adrenals. Another possibility is that the larger adrenals may indicate abnormal corticosteroid production, resulting from disturbed lipid metabolism in EFAD adrenal glands. If corticosteroid production was unimpaired in the EFAD animals it seems unusual that, despite significantly enlarged adrenals, the tissue proliferation in these rats was increased when compared with normal animals. The role of endogenous corticosteroids in modulating chronic and semi-chronic inflammatory models is unclear (33) and this problem is currently under investigation in our laboratory, using metyrapone as a tool.

In conclusion, it would seem that EFAD rats constitute an effective model for studying the roles of endogenous PGs, particularly in acute and chronic inflammation. EFAD is probably more useful, in this context, than PG biosynthesis inhibitors, since these may affect inflammation through mechanisms which do not involve PGs (5).

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