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Effect of cyclosporin A and inhibitors of arachidonic acid metabolism on blood flow and cyclo-oxygenase products in rat skin allografts

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1 Using skin blood flow as a measurement of skin graft rejection in rats, it has been shown that in both isografts and allografts the blood flow at first increases above the normal, after which the flow in isografts returns to normal while that in allografts ceases at the onset of rejection.

2 Cyclosporin A (CSA) 5–40 mg kg^{-1} intramuscularly produced a dose-related inhibition of graft rejection and the pattern of blood flow in the treated allografts became similar to that in isografts in that it remained about 20% above normal throughout the period of treatment.

3 Indomethacin (Indo), inhibitor of cyclo-oxygenase and benoxaprofen (Ben), inhibitor of cyclo-oxygense and lipoxygenase, caused an enhancement of the onset of rejection and an early cessation of blood flow in allografts.

4 The total content of 4 cyclo-oxygenase products (COP), (prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$, 6-oxo-$PGF_{1\alpha}$ and thromboxane B_2 (TxB_2)) increased both in isografts and allografts, but when individual COP were expressed as a percentage of the total, only 6-oxo-$PGF_{1\alpha}$ (the stable metabolite of prostacyclin) increased in allografts.

5 This increased proportion was reduced to normal by a dose of CSA which prolonged graft survival. Indo and Ben partially inhibited COP formation and in particular that of 6-oxo-$PGF_{1\alpha}$. In addition, CSA caused a dose-related inhibition of the prostacyclin produced by zymosan-activated macrophages.

6 These findings in the rat suggest that prostacyclin is partly responsible for the increase in blood flow in allografts prior to rejection; that CSA inhibits both the recruitment of prostacyclin-producing macrophages and prostacyclin formation directly; and that inhibitors of cyclo-oxygenase enhance skin graft rejection by abrogating the immunoregulatory activity of prostacyclin.

Introduction

An inflammatory process involving increased blood flow and vascular permeability frequently takes part in the amplification of an immune response and it seems likely that endogenous substances produced locally are responsible for such vascular changes as, for example, in the graft rejection reaction. We have recently reported changes in blood flow, histamine content and histidine decarboxylase (HDC) activity in rat skin isografts and allografts during the 'healing-in' and rejection reactions. It was also shown that the immunosuppressive agent, cyclosporin A (CSA) which prolongs skin allograft survival, prevents the changes in histamine content and HDC activity in allografts, suggesting that CSA modified the interactions between endothelial cells, lymphocytes and mast cells in graft rejection (Fan & Lewis, 1982).

In view of their effect on vascular reactivity, cyclo-oxygenase products (COP) formed from arachidonic acid (AA) are believed to have important roles in inflammation and tissue repair processes. On the other hand, many observations have suggested possible roles, especially for E-type prostaglandins (PGs), as endogenous modulators of immune responses (for review see Morley, 1981; Lewis, 1983). In an attempt to examine possible functional roles of COP in allograft rejection, we compared the changes in the local levels of four major COP (PGE_2, $PGF_{2\alpha}$, 6-oxo-$PGF_{1\alpha}$ and thromboxane B_2 (TxB_2)) in rat skin isografts and allografts.

The immunosuppressive agent CSA has been shown to inhibit lymphocyte trapping (Ali, Morley & Rumjanek, 1982), recruitment of mononuclear cells

(Rumjanek, Smith & Morley, 1983), and lymphokine production (Alberti, Boraschi, Luini & Tagliabue, 1981; Bunjes, Hardt, Rollinghoff & Wagner, 1981) and it was therefore considered of interest to determine whether CSA modifies the COP levels in allografts during immunosuppression. Furthermore, since indomethacin is known to inhibit COP formation, it has been used in an attempt to determine their importance in allograft reaction. Finally as inhibition of cyclo-oxygenase might enhance the formation of lipoxygenase products, the effects of inhibitors of both enzymes, BW755C and benoxaprofen, have also been examined.

Methods

Animals

Male WAG (RTI^u) and DA (RTI^a) rats were purchased from Bantin & Kingman Ltd. Hull, U.K.

Drugs

Cyclosporin A was a gift from Sandoz AG, Basle. Benoxaprofen and BW755C (3-amino-1[m-(trimoromethyl)-phenyl]-2-pyrazoline) were kindly provided by Lilly Research Centre Ltd. and Wellcome Laboratories respectively.

Radiochemicals

Xenon (^{133}Xe) injection (10mCi in 3 ml), [5,6,8,11,12,14,15 (n)-^3H] PGE_2 (160 Ci mmol^{-1}) and [5,6,8,9,11,12,14,15(n)-^3H] $PGF_{2\alpha}$ (160 Ci mmol^{-1}) were obtained from Amersham International Ltd., Buckinghamshire, U.K.; [5,8,9,11,12,14,15(n)-^3H] 6-oxo-$PGF_{1\alpha}$ (120 Ci mmol^{-1}) and [5,6,8,9,11,12,14,15(n)-^3H] TxB_2 (125 Ci mmol^{-1}) were obtained from New England Nuclear, Boston, Mass., USA.

Skin graft procedure

The Rats weighing 150–200 g were anaesthetized with 0.25 ml kg^{-1} Hypnorm (0.315 mg ml^{-1} fentamyl citrate and 10 mg ml^{-1} fluanisone, Janssen). Full thickness skin grafts (2 cm × 2 cm) were removed from the abdomen of donor rats and transplanted onto dorsal graft beds of the recipients by means of interrupted 5/0 silk suture. Each WAG rat received two skin grafts from DA (allogeneic) or WAG (isogeneic) rats.

Blood flow measurement

Blood flow measurements were carried out on conscious rats restrained in a modified Bollman cage, using a ^{133}Xe clearance technique. The room temperature was kept at 20 ± 1°C. A constant microinjection of 10 µl ^{133}Xe in saline was made intradermally to each graft on alternate days. The washout of radioactivity from the skin graft was monitored using a collimated γ-scintillation detector for 6 min; the radioactivity being recorded every 40 s and printed automatically on an SR7 scaler ratemeter (Nuclear Enterprises Ltd. Reading) coupled to an ESP 401 α-numeric and graphic printer (English Numbering Machines Ltd. London). The clearance of ^{133}Xe at 6 min was calculated as a percentage of the initial count. This provides a rapid estimation of skin blood flow as shown earlier (Fan & Lewis, 1981).

Calculation of mean survival times of skin allografts

In normal skin, 65–72% of the ^{133}Xe-saline injected intradermally was cleared after 6 min. Healing, represented by establishment of blood flow, was usually completed by day 4 after grafting. After 9 days, the skin grafts failed to clear more than 50% of the injected radioactivity and since this change was irreversible, the grafts were regarded as being rejected. The survival time of the allografts in each treatment group was pooled and calculated to give a mean survival time ± s.e.mean.

Preparation of samples for measurements of cyclo-oxygenase products

At various times after grafting, rats were killed by cervical dislocation and the graft was excised and immediately frozen on dry ice. During the first few days after transplantation, the graft could readily be lifted from its wound bed. At later stages, it had to be dissected free. Therefore, grafts were always removed with the subcutaneous tissue so that meaningful comparisons could be made. The grafts were weighed after removal and stored at −20°C until they were extracted. Samples of normal ungrafted abdominal skin were stored and treated in the same manner as the grafts. In order to prepare extracts, the tissues were thawed, suspended in 5 ml of ice-cold phosphate-buffered saline (PBS) and homogenized at 4°C with a pestle and mortar, a small amount of cleaned sand being added to facilitate the procedure. The pestle and mortar were washed with a further 2 ml of PBS and the washings added to the homogenate. The homogenates were centrifuged at 2,500 r.p.m. with the MSE GF8 centrifuge for 10 min. The supernatants were centrifuged again at 30,000 *g* (4°C, 20 min) with a Sorvall Superspeed RC2-B automatic refrigerated centrifuge. Two ml of the clear supernatant fluids thus obtained were acidified with 0.1 M HCl to pH 2–3 to precipitate the

protein. After centrifugation (2,500 r.p.m. for 10 min), prostaglandins or TxB_2 in the samples were extracted by adding 8 ml of diethyl ether to 2 ml of the deproteinized samples in glass extraction tubes, shaking and recovering the organic phase. This was done twice, the diethyl ether pooled together and evaporated to dryness. The dried extracts were dissolved in 2 ml tricine (N-tris(hydroxymethyl) methyglycine) buffered saline and the contents of prostaglandins or TxB_2 determined by radioimmunoassay as described below. Since there was no significant difference between unextracted and extracted samples, most samples were assayed unextracted.

Preparation of rat peritoneal macrophages

A group of four WAG rats were killed by cervical dislocation. Twenty ml of sterile Dulbecco's modified Eagles medium (DMEM: Gibco) containing antibiotics (100 units penicillin ml^{-1} and 100 μg streptomycin ml^{-1}: Gibco) was injected intraperitoneally. The abdomen was massaged gently for 1 min to ensure good circulation of the medium in the peritoneum. The abdomen was then shaved, cleaned with 70% ethanol and an incision was made through which the fluid containing peritoneal cells was withdrawn using a sterile syringe. The cells were centrifuged (100 *g*, 15 min), pooled, washed twice and resuspended in DMEM containing 5% heat-inactivated (56°C, 30 min) foetal calf serum (referred to as culture medium hereafter).

Total cell counts and cell viability

Cell viability was determined using the trypan blue exclusion method; 100 μl of cell suspension was mixed with 100 μl of trypan blue dye (Gibco) and 400 μl of DMEM. After 5 min at room temperature the number of viable cells that had excluded the dye were counted in an improved Neubauer haemocytometer chamber. The cell suspension was readjusted according to the number of live cells to obtain a concentration of 3.2×10^6 viable cells per ml.

Preparation of serum-opsonized zymosan

Zymosan (Grade A from *S. ceravisiae* yeast, Sigma Chemical Co.) was washed three times with 0.9% w/v sterile NaCl solution (saline), then sterilized by autoclaving at 15 psi for 15 min, allowed to cool and the saline removed by centrifugation (1,000 *g*, 5 min).

Rat blood was obtained by cannulation of the descending aorta. The blood was allowed to clot and centrifuged for 5 min at 1,000 *g*. The serum was collected carefully and sterilized by passage through a 0.22 μm millipore filter (Millex). The sterilized zymosan was then resuspended in fresh rat serum at a concentration of 1 mg ml^{-1} and incubated at 37°C for 30 min. The serum was then removed by centrifugation and the opsonized zymosan was prepared for each experiment.

Culturing techniques

Linbro 24-well culture plates were used. All manipulations with cells were carried out in a laminar flow sterile cabinet (Microflow Pathfinder Ltd., Hampshire). Cell suspension in a volume of 500 μl (containing 1.6×10^6 viable cells) was pipetted into each well (2 cm^2). The plates were then placed in an incubator at 37°C to allow the cells to adhere. After 2 h, the non-adherent cells were removed by vigorous washing with fresh culture medium. The adherent cells were re-incubated for 24 h before the culture medium was renewed.

Using a similar method it has been shown that more than 90% of the adherent cells were macrophages on the basis of their ability to phagocytose IgG-coated sheep red blood cells, the presence of Fc receptor sites and the presence of granules staining for non-specific esterase (Ford-Hutchinson & Doig, 1979).

After removal of culture medium at 24 h, 400 μl culture medium was added, followed by 50 μl of serum-opsonized zymosan. Finally 50 μl of cyclosporin A (CSA) or the vehicle control was added to make up a total volume of 500 μl. The culture plates were returned to the incubator for 48 h. After this period, the supernatant from each well was collected and centrifuged to remove the zymosan and cell debris. The clear supernatants were assayed for PGE_2, $PGF_{2\alpha}$, 6-oxo-$PGF_{1\alpha}$ and TxB_2 using radioimmunoassay. Occasionally, cell viability at the end of experiments was examined using the trypan blue exclusion method. At the highest concentration of CSA (3 μg ml^{-1}), over 80% of the cells were alive.

Analysis of COP contents by radioimmunoassay (RIA)

The samples were subjected to RIA by the methods described by Jose, Niederhauser, Piper, Robinson & Smith (1976). The assay procedures were similar for the four COP assayed: PGE_2, $PGF_{2\alpha}$, 6-oxo-$PGF_{1\alpha}$ and TxB_2. Sheep anti-PGE_2 antiserum and rabbit anti-$PGF_{2\alpha}$ antiserum were produced by Dr P.J. Jose of this department. Since prostacyclin (PGI_2) and thromboxane A_2 (TxA_2) have such short half-lives, these levels were measured in terms of their stable metabolites 6-oxo-$PGF_{1\alpha}$ and TxB_2 respectively. Sheep anti-6-oxo-$PGF_{1\alpha}$ antiserum was provided by Dr L. Myatt of Hammersmith Hospital, London.

Table 1 Cross-reactions of prostaglandins, thromboxane B_2 (TxB_2) and fatty acids to the specific antisera used in the present study

Test substance	*% cross reactivity* of test substance with: Anti-PGE_2*	*Anti-$PGF_{2\alpha}$*	*Anti-6-oxo-$PGF_{1\alpha}$*	*Anti-TxB_2*
PGA_2	0.36	0.01	0.007	
PGB_2	0.04	0.01		
PGD_2	0.04	0.02		
PGE_1	26.00	—	0.1	
PGE_2	100.00	0.03	0.007	0.02
$PGF_{1\alpha}$	1.2	18.00	0.08	
$PGF_{2\alpha}$	1.4	100.00	0.15	0.1
6-oxo-$PGF_{1\alpha}$	0.03	0.18	100.00	0.01
TxB_2	0.03	0.08	0.007	100.00
Arachidonic acid	0.06	0.01	0.01	
12-HETE	0.03	0.01		
Oleic acid	0.002	0.001		

* Cross reactivity (in %) calculated from amount of prostaglandins or fatty acid required to reduce the binding of [^{3}H]-prostaglandins or [^{3}H]-TxB_2 to homologous antiserum by 50%.

Rabbit anti-TxB_2 antiserum was a gift from Dr J.B. Smith of Thomas Jefferson University, Philadelphia, U.S.A. These antisera were shown to have good specificity, as indicated in Table 1. Routine checks were carried out to provide a guide-line for the dilution of the antiserum so that in the absence of unlabelled prostaglandins or TxB_2 45–50% of the tritiated prostaglandins or TxB_2 was bound.

Statistical analysis

Results are given as the mean ± s.e.mean of n animals. Comparisons between groups were made with two-tailed Student's t test for unpaired data. Isografts or treated allografts were always compared with untreated allografts. The significance levels of difference were indicated by P (probability) values in the Tables and Figures. Where no P value is indicated, there was no significant difference between the groups.

Results

Blood flow changes in rat skin allografts and isografts

The pattern of blood flow changes in allografts and isografts is illustrated in Figure 1. The blood flow in both types of grafts increased during the first few days after grafting and a good flow was established by day 4. It gradually increased to a maximum until by day 7 it was about 20% higher than normal skin blood flow in isografts and about 10% higher in allografts. The blood flow in allografts began to decrease at day 8 and fell below the 50% clearance level by day 9. The maximum blood flow in isografts was maintained for 5–6 days before returning to the normal range.

Effects of drugs on blood flow changes

(a) *Cyclosporin A (CSA)* Four doses of CSA were studied: 5,10,20 and 40 mg kg^{-1} given daily either until the grafts were rejected or up to a maximum of 14 days. Table 2 shows a dose-dependent effect of CSA in prolonging allograft survival. The lowest dose of 5 mg kg^{-1} was marginally effective delaying rejection until 12 days after grafting, while the highest dose of 40 mg kg^{-1} maintained the viability of the allografts for at least 50 days.

CSA-induced suppression of skin graft rejection is reflected by its effect on blood flow as shown in Figure 1. The allografts in rats treated with CSA 20 mg kg^{-1} daily behaved like isografts both

Table 2 Effect of cyclosporin A (CSA) on rat skin allograft survival time

Group	n	*Mean survival time (days ± s.e.mean)*
Control, no treatment	30	9.4 ± 0.1
Control, olive oil (0.1 ml)	6	9.6 ± 0.2
CSA (5 mg kg^{-1} daily)	4	12.3 ± 0.4
CSA (10 mg kg^{-1} daily)	6	23.5 ± 2.0**
CSA (20 mg kg^{-1} daily)	10	34.7 ± 0.4**
CSA (40 mg kg^{-1} daily)	4	54.0 ± 0.9**

CSA (0.1 ml, at various concentrations) or olive oil was injected intramuscularly once daily either until the grafts were rejected or up to a maximum of 14 days.
** $P < 0.001$.

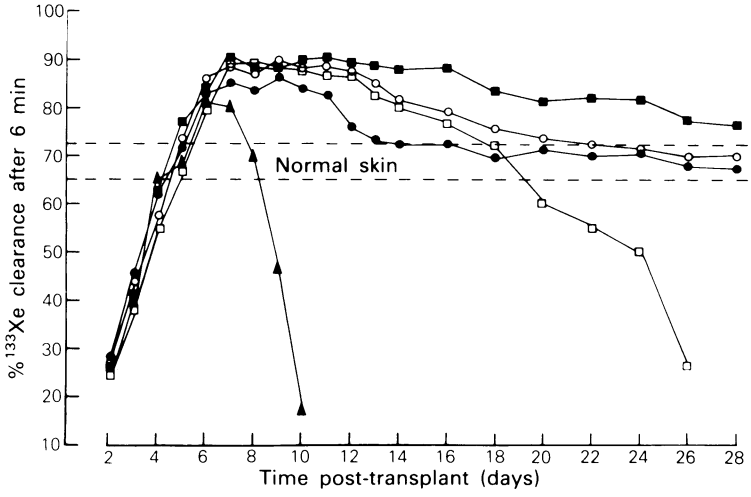

Figure 1 Blood flow changes in rat skin isografts (●) and allografts (▲) plotted against number of days after grafting. Each point is the mean of at least six animals. No blood flow could be detected in the grafts by day 2. Normal blood flow was established in all grafts by day 5. In isografts the blood flow increased forming a plateau at about 20% above the normal skin flow between day 7 to 11 and subsequently decreased to normal skin blood flow. In allografts the plateau of active blood flow was maintained for 48 h only, after which it fell, with ^{133}Xe clearance below 50% around day 9. The blood flow pattern of the cyclosporin A (CSA)-treated group resembled that observed in isografts rather than untreated allografts, and remained above the normal skin blood flow throughout the period of treatment (14 days). CSA 10 mg kg^{-1} daily (□) was able to maintain active blood flow up to 18 days and the skin grafts and were rejected by about 24 days. CSA 20 mg kg^{-1} daily prolonged allograft survival to 35 days (■) but produced no apparent effect on isografts (○).

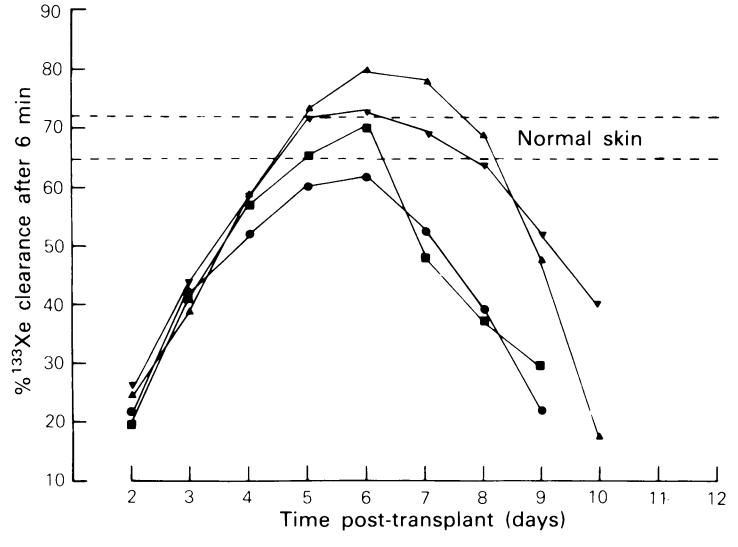

Figure 2 Comparison of the effect of indomethacin (Indo) (●), benoxaprofen (Ben) (■) and BW755C (○) on allograft blood flow. Control (untreated) allograft blood flow is shown by (▲). Drugs were given orally: Indo (2 × 1.5 mg kg^{-1} daily, $n = 6$), Ben (2 × 15 mg kg^{-1} daily, $n = 8$) and BW755C (2 × 25 mg kg^{-1} daily, $n = 6$). Both Indo and Ben suppressed the maximum blood flow in allografts, and this was associated with accelerated rejection (Indo 7.1 ± 0.6, Ben 6.9 ± 1.1 days). BW755C produced only a marginal suppression of maximum blood flow and did not affect the rejection time. Note different time scales in Figures 1 and 2.

Table 3 Effect of oral administration of indomethacin, benoxaprofen or BW755C on rat skin allograft survival

Group	n	*Mean survival time (days ± s.e.mean)*
Control, no treatment	30	9.4 ± 0.1
Vehicle control, syrup	6	9.2 ± 0.2
Indomethacin (2×0.5 mg kg^{-1} daily)	6	9.3 ± 0.3
Indomethacin (2×1.5 mg kg^{-1} daily)	6	7.1 ± 0.6**
Vehicle control, carboxylmethyl cellulose	6	9.3 ± 0.6
Benoxaprofen (2×5 mg kg^{-1} daily)	4	9.3 ± 0.3
Benoxaprofen (2×10 mg kg^{-1} daily)	6	8.4 ± 1.2
Benoxaprofen (2×15 mg kg^{-1} daily)	8	6.9 ± 1.1**
Vehicle control, saline	6	9.4 ± 0.2
BW755C (2×25 mg kg^{-1} daily)	10	9.6 ± 0.7
BW755C (2×50 mg kg^{-1} daily)	6	9.5 ± 0.5

Drugs were given twice daily until the grafts were rejected.
** $P < 0.001$.

during the 'healing-in' process (day 2–5) and subsequently when the blood flow remained about 20% above that in normal skin, throughout the period of treatment.

(b) *Indomethacin* Daily oral administration of 2×0.5 mg kg^{-1} indomethacin did not produce any obvious changes in the blood flow pattern in allografts. The treated allografts were rejected at 9.3 ± 0.3 days (9.4 ± 0.1 days in control group). However, similar administration of a larger dose of indomethacin (2×1.5 mg kg^{-1}) resulted in accelerated allograft rejection (7.1 ± 0.6 days) as shown in Figure 2. The maximum blood flow achieved in these grafts, unlike that observed in untreated allografts, was never above that of normal skin. This finding suggested that prostaglandins might be involved in the vasodilation which occurred before rejection.

Higher doses (2×2.5 mg kg^{-1} and 2×3.5 mg kg^{-1} daily) proved toxic to rats receiving allografts, most rats dying before day 7. Therefore, it was not possible to establish a dose-dependent effect of indomethacin on allograft survival. The results are summarized in Table 3.

(c) *Benoxaprofen* The effect of inhibition of the lipoxygenase as well as the cyclo-oxygenase pathway of arachidonic acid metabolism on allograft survival was studied using benoxaprofen. Daily oral administration of 2×5 mg kg^{-1} benoxaprofen produced no apparent effect on the blood flow and the survival time of allografts (Table 3). When a higher dose (2×10 mg kg^{-1} daily) was used, there was a marginal acceleration of graft rejection (8.4 ± 1.2 days versus 9.3 ± 0.6 days in vehicle control group). The highest dose tested, 2×15 mg kg^{-1} daily, was able to cause a further enhancement of rejection to 6.9 ± 1.1 days and a reduction in maximal blood flow as shown in Figure 2.

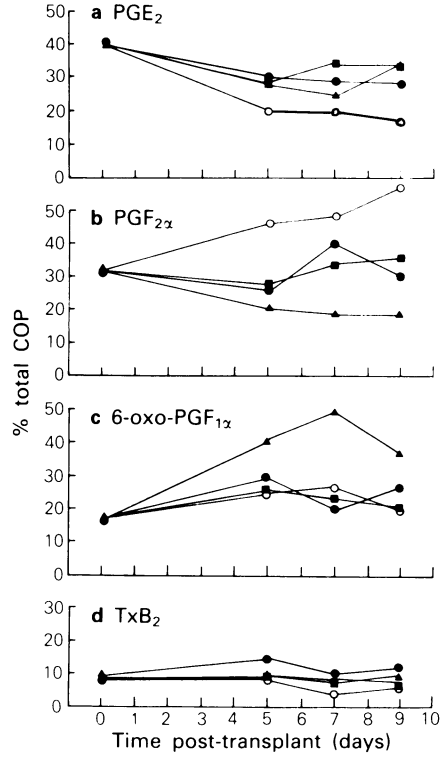

Figure 3 Proportion of (a) prostaglandin E_2 (PGE_2), (b) $PGF_{2\alpha}$, (c) 6-oxo-$PGF_{1\alpha}$ and (d) thromboxane B_2 (TxB_2) expressed as a percentage of total cyclo-oxygenase products (COP) in isografts (●), isografts treated with cyclosporin A (CSA) 20 mg kg^{-1} intramuscularly daily (○), allografts (▲) and allografts treated with CSA 20 mg kg^{-1} intramuscularly daily (■). Data represent means of at least five animals.

Table 4 Cyclo-oxygenase products (COP) in isografts (I) and allografts (A)

Days after grafting	*PGE_2* (ng g^{-1})		*$PGF_{2\alpha}$* (ng g^{-1})		*PGI_2 (6-oxo-$PGF_{1\alpha}$)* (ng g^{-1})		*TxA_2 (TxB_2)* (ng g^{-1})		*Total COP* (ng g^{-1})	
	I	A	I	A	I	A	I	A	I	A
0	155±14	148±15	136±7	106±4	58±11	73±14	39±3	31±7	388±35	358±40
1	204±45	171±260	185±53	256±47	62±10	182±22	33±33	60±1	484±111	669±96
3	232±32	278±31	300±51	211±33	199±25	359±79	108±20	122±30	930±128	970±96
5	395±54	378±31	341±44	296±66	394±49	555±32	197±13	129±9	1297±160	1351±173
7	350±47	357±46	495±26	269±42	238±49	716±25	115±13	115±6	1198±129	1457±119
9	463±94	532±92	492±68	291±37	433±69	560±59	196±30	138±43	1584±261	1521±233

Contents of individual cyclo-oxygenase products in skin graft extracts (ng g^{-1} tissue) were measured by radioimmunoassays using the antisera described in Table 1.
Data represent mean ± s.e.mean of at least 5 animals.

(d) *BW755C* 3-Amino-1-(m-(trifluoromethyl)-phenyl)-2-pyrazoline (BW755C) is also an inhibitor of both cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism (Higgs, Flower & Vane, 1979). Administration of 50 mg kg^{-1} (2 × 25 mg kg^{-1} daily, p.o.) as shown in Figure 2 did not alter allograft rejection. However, the maximum blood flow which normally occurs on day 7 in untreated allografts, appeared to be suppressed by the drug treatment.

Cyclo-oxygenase products (COP) in skin grafts

The contents of the four individual COP in isografts and allografts at different times after grafting are given in Table 4. There were similar increases of total COP in isografts and allografts, but there were significant differences in the profile of some individual COP, particularly in 6-oxo-$PGF_{1\alpha}$. When the concentrations of individual prostanoids were expressed as a percentage of the total COP it was clear that 6-oxo-$PGF_{1\alpha}$ was the only metabolite to increase in allografts as shown in Figure 3.

Prostaglandin E_2 In isografts and untreated allografts, PGE_2 levels changed in a similar way from day 1 to 9. On day 7, the PGE_2 content was elevated 2-fold that of normal skin, from 155 ± 14 to 350 ± 47 ng g^{-1} tissue in isografts, and from 148 ± 15 to 357 ± 46 ng g^{-1} tissue in allografts.

In normal skin, PGE_2 constituted about 40% of the total COP. On day 7 it was 29% and 25% in isografts and allografts respectively.

Prostaglandin $F_{2\alpha}$ The $PGF_{2\alpha}$ levels in both isografts and allografts increased after transplantation. In isografts, there was a 3.5-fold increase in $PGF_{2\alpha}$ on day 7, from 136 ± 7 to 495 ± 26 ng g^{-1} tissue; while in allografts the increase was 2.5 times that of normal skin, from 106 ± 4 to 269 ± 42 ng g^{-1} tissue. At this time, $PGF_{2\alpha}$ accounted for 41% of the total COP in isografts while it was only 18% of the total COP in allografts. In normal skin, $PGF_{2\alpha}$ constituted 29–35% of total COP.

6-oxo-Prostaglandin $F_{1\alpha}$ There was an increase of 6-oxo-$PGF_{1\alpha}$ in both isografts and allografts. However, there was a significant difference between the 6-oxo-$PGF_{1\alpha}$ levels in the two types of grafts. For example on day 7, the 6-oxo-$PGF_{1\alpha}$ levels in isografts was increased 4-fold, from 58 ± 11 to 238 ± 49 ng g^{-1} tissue; while that in allografts was elevated 10-fold from 73 ± 14 to 716 ± 25 ng g^{-1} tissue.

The 6-oxo-$PGF_{1\alpha}$ content in normal skin accounts for only 15–20% of the total COP. On day 7, it remained at 20% in isografts but was 49% in allografts.

Table 5 Modification of total cyclo-oxygenase products expressed as ng g^{-1} tissue in rat skin grafts by cyclosporin A (CSA, 20 mg kg^{-1} daily), indomethacin (Indo, 2 × 1.5 mg kg^{-1} daily) and benoxaprofen (Ben, 2 × 15 mg kg^{-1} daily)

Type of graft	*Days after grafting*			
	0	5	7	9
Isograft	388 ± 35	1297 ± 160	1198 ± 129	1585 ± 261
Isograft + CSA		501 ± 89**	840 ± 111	693 ± 92**
Allograft	358 ± 40	1351 ± 173	1457 ± 119	1521 ± 233
Allograft + CSA		877 ± 70	855 ± 103**	850 ± 49*
Allograft + Indo		661 ± 132*	999 ± 155*	769 ± 154*
Allograft + Ben		1358 ± 135	1180 ± 168	1256 ± 94

* $P < 0.01$, ** $P < 0.005$.
Data represent mean ± s.e.mean of 2 grafts from 5–7 animals.

Thromboxane B_2 Although there was a general rise in TxB_2 content in isografts and allografts, there was no significant change in the proportion of TxB_2 in the two types of grafts. In normal skin, TxB_2 accounts for only 10% of total COP. On day 7, it remained at 10% in isografts and 8% in allografts.

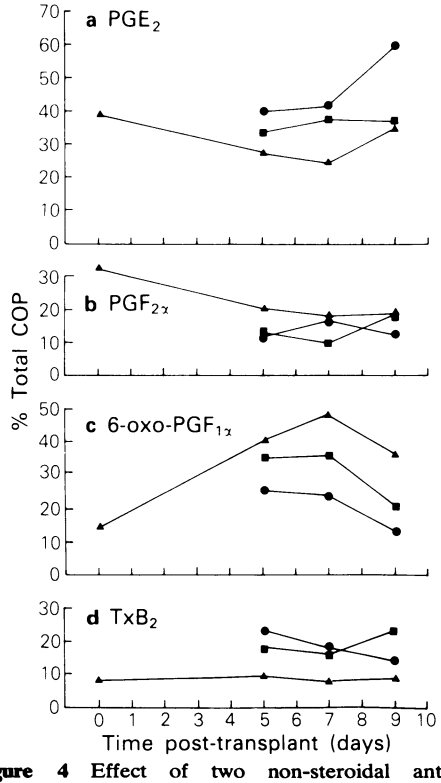

Figure 4 Effect of two non-steroidal anti-inflammatory agents on the proportion of prostaglandins (PGs) and thromboxane B_2 (TxB_2) as % of total cyclo-oxygenase products (COP) in skin allografts (▲). (●) Indomethacin (Indo), 2 × 1.5 mg kg^{-1} daily. (■) Benoxaprofen (Ben), 2 × 15 mg kg^{-1} daily. Data represent means of at least five animals.

Effect of CSA on the levels of COP in isografts

At 20 mg kg^{-1} daily, CSA inhibited the increase in the total COP in isografts normally observed between days 5–9 post-transplant (Table 5). As shown in Figure 3, while the proportion of PGE_2, 6-oxo-$PGF_{1\alpha}$ and TxB_2 as a percentage of total COP was reduced by CSA, that of $PGF_{2\alpha}$ was increased to a maximum of 58% on day 9.

Effects of CSA, indomethacin and benoxaprofen on the levels of COP in allografts

Cyclosporin A (CSA) The effects of CSA at a dose which prolongs allograft survival, i.e. 20 mg kg^{-1} on the profile of COP in allografts, are summarized in Table 5. It is clear that CSA has a profound effect on the COP levels in allografts. It greatly reduced the increase in the total COP in allografts normally observed between day 5 and day 9 after grafting. The levels of PGE_2, 6-oxo-$PGF_{1\alpha}$ and TxB_2, but not that of $PGF_{2\alpha}$, were all reduced. The most outstanding change was the proportion of total COP as 6-oxo-$PGF_{1\alpha}$, as illustrated in Figure 3. It was reduced to a level comparable with that observed in normal skin and isografts.

Indomethacin Table 5 shows that on day 7, indomethacin caused approximately 30% inhibition of total COP, from 1457 ± 119 to 999 ± 155 ng g^{-1} tissue, and a similar degree of inhibition was maintained up to day 9. Indomethacin produced a selective suppression of $PGF_{2\alpha}$ from 269 ± 42 to 179 ± 24 ng g^{-1} tissue on day 7 and 6-oxo-$PGF_{1\alpha}$ from 716 ± 25 to 235 ± 32 ng g^{-1} tissue on day 7, but apparently had no effect on PGE_2 and TxB_2 levels. Figure 4 shows the effect of indomethacin on the COP expressed as a percentage of the total COP. There is no clear explanation for this finding.

Benoxaprofen The fact that benoxaprofen also accelerated allograft rejection in a way indistinguishable from indomethacin prompted us to examine whether

benoxaprofen caused inhibition of COP production as well. Benoxaprofen did not produce any significant changes in the total COP when compared with those in untreated rats (Table 5). However, it was found to cause a moderate inhibition of 6-oxo-$PGF_{1\alpha}$ from 716 ± 25 to 424 ± 46 ng g^{-1} tissue on day 7 (Figure 4).

Cyclo-oxygenase products in rat peritoneal macrophages

The amounts of each prostaglandin and TxB_2 released by activated rat peritoneal macrophages as measured using radioimmunoassays are shown in Table 6. The major COP released by these macrophages was 6-oxo-$PGF_{1\alpha}$. These observations were similar to those of Doig (1981). Using a quantitative gas chromatography- mass spectrometry assay, she found that 228 ± 42 ng of 6-oxo-$PGF_{1\alpha}$ was produced per 2×10^7 cells seeded. In the present study a lower number of cells (i.e. 1.6×10^6) was used and they produced a proportionate amount of 6-oxo-$PGF_{1\alpha}$ i.e. 17.5 ± 1.2 ng.

When CSA was added to the culture, it produced a dose-related inhibition of 6-oxo-$PGF_{1\alpha}$ release by the macrophages activated by zymosan as shown in Table 7. At the end of the experiment, the cells were still viable (80%), indicating that CSA at the highest concentration used (3 μg ml^{-1}) was not cytotoxic.

Discussion

Several earlier studies on various allograft models have shown elevated levels of PGE during rejection (Anderson, Newton & Jaffe, 1975). However, the possible implications of these findings in relation to the vascular events within the grafted tissue have not been fully considered.

Lewis & Mangham (1978) found in the rabbit that the PGE_2 levels in skin grafts were low during the initial healing-in process. In autografts it remained low, but rose sharply in allografts during the peak of high blood flow. The $PGF_{2\alpha}$ levels rose only slightly during healing and rejection. Systemic treatment with indomethacin produced no apparent effect on graft survival but caused a decrease in the maximum blood flow in the allografts. These observations suggested that a prostaglandin, probably PGE_2, was partially responsible for the increased blood flow at its peak level in rabbit skin allografts.

Table 6 The amounts of prostaglandins and thromboxane B_2 (TxB_2) released by unelicited peritoneal macrophages after 48 h incubation with zymosan (50 μg ml^{-1})

Type of COP	(ng per 1.6×10^6 cells)
PGE_2	7.2 ± 0.9
$PGF_{2\alpha}$	3.6 ± 0.8
6-oxo-$PGF_{1\alpha}$	17.5 ± 1.2
TxB_2	2.4 ± 0.3

Data represent mean ± s.e.mean from 3 experiments.

Table 7 Inhibitory effects of cyclosporin A (CSA) on the production of 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) by zymosan-activated rat peritoneal macrophages

CSA (μg ml^{-1})	*6-oxo-$PGF_{1\alpha}$* (ng/1.6×10^6 cells)	*% inhibition*
0	17.5 ± 1.2	—
0.1	15.8 ± 2.1	9.7
0.3	16.0 ± 1.1	8.6
1.0	10.4 ± 1.6	40.6*
3.0	6.6 ± 0.9	62.3*

Data represent mean ± s.e.mean from 3 expreiments
*$P < 0.01$.

In the present investigation, we examined whether similar changes occur in rat skin grafts. In addition to PGE_2 and $PGF_{2\alpha}$, the levels of 6-oxo-$PGF_{1\alpha}$ and TxB_2 were also monitored to see if the relative changes of arachidonic acid metabolites were related to the blood flow changes in isografts and allografts.

Although there was an increase in total COP in both isografts and allografts, the most significant increase of individual COP was that of 6-oxo-$PGF_{1\alpha}$. Not only did it increase almost 10-fold during the allograft reaction, but when expressed as a percentage of total COP, 6-oxo-$PGF_{1\alpha}$ increased from about 20% to 50%.

Furthermore, both indomethacin and benoxaprofen caused a reduction in 6-oxo-$PGF_{1\alpha}$ production and also reduced the peak blood flow in allografts. Thus it appears that PGI_2 might well be responsible for part of the hyperaemia produced in rat skin allografts before rejection, just as it had been found earlier that PGE_2 was probably responsible for a similar effect in rabbits (Lewis & Mangham, 1978).

There are two possible explanations of the indomethacin-induced enhancement of rejection. Firstly, since PGI_2 appears to be at least partly responsible for maintaining a maximal blood flow in the skin grafts at day 5 to 7, removal of this vasodilatation by indomethacin might itself enhance the vascular shutdown which occurs at the onset of rejection. Secondly the view is emerging that certain prostaglandins, in particular PGE_2 and PGI_2, are capable of regulating lymphocyte function (Lewis, 1983). This view is based on the observations that the PGEs

inhibit mitogen-induced lymphocyte transformation (Smith, Steiner & Parker, 1971), lymphocyte-mediated cytotoxicity (Henney, Bourne & Lichtenstein, 1972), antibody production (Melmon, Bourne, Weinstein, Shearer, Kram & Bauminger, 1974) and lymphokine secretion (Gordon, Bray & Morley, 1976; Rappaport & Dodge, 1982; Walker, Kristensen, Bettens & de Weck, 1983). More recently Mertin & Stackpoole (1981) showed that anti-PGE antisera significantly suppressed cell-mediated immune responses *in vivo*, further suggesting the importance of PGEs as extracellular modulators of T cell functions.

The mechanism by which PGEs and probably PGI_2 act as negative immuno-regulatory agents to inhibit immune responses is possibly associated with the induction of suppressor T cells (Fischer, Durandy & Griscelli, 1981). The generation of these cells would normally counter-balance the activity of helper T cells so as to adjust the intensity of an immune response. Pharmacological intervention of the immunoregulatory activities of prostaglandins by cyclo-oxygenase inhibitors has been shown to inhibit both the generation and effector function of immunoregulatory suppressor cells or their precursors whilst leaving T helper and B cell function unimpaired (Goodwin, Bankhurst & Messner, 1977; Nicklin & Shand, 1982). They also enhance the production of lymphokines such as interleukin 2 (Rappaport & Dodge, 1982; Walker *et al.*, 1983). The cyclo-oxygenase inhibitors would therefore tend to potentiate the immune response which could explain the present finding of enhancement of rejection. Consistent with this view are the findings that long-acting prostaglandins prolong the survival of murine skin allografts (Anderson, Jaffe & Graff, 1977) and rat cardiac allografts (Strom & Carpenter, 1980). In addition, Kort, Bonta, Adolfs & Westbroek (1982) have shown a synergism of a long-acting PGE, with azathioprine or prednisolone on the survival of heart allografts in rats.

The immunosuppressive cyclosporin A (CSA) also reduced COP production in the skin grafts. However, the effect of this drug on blood flow was quite different from that of the cyclo-oxygenase inhibitors. The blood flow in skin allografts of rats treated with CSA, like isografts, increased to a level about 20% greater than normal skin blood flow and remained elevated throughout the treatment period. This vasodilatation was not due to COP, since their production was significantly reduced by CSA. However, it seems unlikely that the inhibition of COP formation by CSA is responsible for its immunosuppressive activity since the cyclo-oxygenase inhibitor indomethacin enhanced graft rejection while suppressing COP formation.

There are at least two ways in which CSA might influence the formation of prostaglandins in rat skin grafts. Firstly, it might prevent the recruitment into the allografts of the mononuclear cells which are likely to be responsible for the PGI_2 formation. Rumjanek *et al.* (1983) found that CSA was able to inhibit Con A-induced but not carrageenan-induced accumulation of ^{51}Cr-labelled mononuclear cells in rats. These findings imply that the inhibition of mononuclear cells into a site like a skin graft was not simply the result of a general cytotoxic effect of CSA on circulating mononuclear cells. In addition, it was obvious even from a cursory histological examination of the skin allografts in the present investigation that cell migration was considerably less in the CSA-treated than the untreated rats. Secondly, since CSA is known to inhibit release of interleukins from mononuclear cells (Bunjes *et al.*, 1981; Alberti *et al.*, 1981; Gordon, Nouri & Thomas, 1982), it is possible that the immunosuppressive agent inhibited prostaglandin formation by the mononuclear cells directly. When rat peritoneal macrophages were stimulated with zymosan in the present experiments, it was found that they produced COP and that this production was reduced 62% by CSA without showing any evidence of cytotoxicity. Therefore, it seems likely that CSA affects COP formation both indirectly by reducing infiltration of mononuclear cells and directly by inhibition of macrophage-derived COP. The fact that CSA also suppresses COP formation in skin isografts would indicate that it has anti-inflammatory activities in addition to its immunosuppressive activity.

Our previous study (Fan & Lewis, 1982) and the present investigation illustrate the multiplicity of activities and sources of histamine and prostaglandins in both non-immune and immunologically-induced inflammation. The vascular events during allograft rejection are likely to involve complex interactions between these vasoactive chemical mediators and mononuclear cell-derived interleukins and such interactions might well affect the ultimate course of some immunological reactions. Further efforts to analyse the various components of the immune response with more specific inhibitors or antagonists may identify possible sites for pharmacological intervention and provide avenues for a better immunopharmacological control of transplant rejection.

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