Inflammatory mechanisms in the passive cutaneous anaphylactic reaction in the rabbit: evidence that novel mediators are involved

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1 We have examined the mechanisms of local oedema formation in the passive cutaneous anaphylactic (PCA) reaction in the rabbit.

2 IgE-containing antiserum was injected i.d. and allowed to sensitize skin sites for periods up to 240 h. Antigen (bovine gamma globulin) was injected i.d. or i.v. and local oedema formation assessed by the accumulation of i.v. injected ¹²⁵I-labelled rabbit serum albumin. Potential inhibitors were mixed with antigen prior to i.d. injection or were administered i.v.

3 Maximum oedema formation was observed when a sensitization period of 48–72 h was used. Oedema formation in the PCA reaction was of short duration with a *t*_{1/2} of approximately 15 min. No evidence of late oedema formation (up to 6 h) was found.

4 Local oedema formation in the PCA was reduced by indomethacin suggesting that vasodilator, oedema-potentiating prostaglandins were released. However, it was likely that other vasodilators were also generated.

5 Antihistamines were poor inhibitors of oedema formation as were PAF antagonists, a 5-lipoxygenase inhibitor, a kallikrein inhibitor, a bradykinin antagonist and anti-C5a antibody.

6 Local oedema formation in the PCA was partially reduced by neutrophil depletion and colchicine suggesting that neutrophil-dependent mediators were involved.

7 Exudate fluid from anaphylactic reactions in the rabbit peritoneal cavity contained permeability-increasing activity when injected into rabbit skin. This activity is now being characterized.

8 A vasodilator prostaglandin appears to be released in the rabbit PCA reaction but none of the established permeability-increasing mediators appears to be involved. Thus, there may be novel inflammatory mediators generated in this reaction which may have relevance for human allergic skin diseases.

Keywords: Cutaneous anaphylaxis; oedema formation; IgE; peritoneal anaphylaxis; rabbit skin; prostaglandins; PAF; histamine; neutrophil-dependent oedema

Introduction

One of the characteristic features of allergic inflammation is a local increase in microvascular permeability in response to locally generated mediators (Burke & Miles, 1958). Oedema formation in skin is dependent on a synergism between increased vascular permeability and increased blood flow (Williams and Morley, 1973; Williams & Peck, 1977). We have previously investigated the mediators responsible for the oedema formation in rabbit skin observed in the reversed passive Arthus reaction (Hellewell & Williams, 1986; Williams *et al.*, 1986; Hellewell *et al.*, 1988) and in response to intradermal injection of yeast cell walls (zymosan) (Wedmore & Williams, 1981; Williams & Jose, 1981) and synthetic cationic proteins (Needham *et al.*, 1988). In all these reactions, there was evidence for vasodilator prostaglandin production as indicated by reduced oedema formation in the presence of indomethacin. The complement activation fragment C5a, or its des Arg metabolite, is a major permeability-increasing agent in the Arthus reaction and in the response to zymosan but anaphylatoxic histamine release by C5a is relatively unimportant in these reactions (Williams & Jose, 1981; Williams *et al.*, 1986; Hellewell *et al.*, 1988). Platelet activating factor (PAF) also plays a significant role in oedema formation in the Arthus reaction (Hellewell & Williams, 1986; Warren *et al.*, 1989; Hellewell, 1990).

The passive cutaneous anaphylactic (PCA) reaction is a model of allergic inflammation characterized by local oedema formation in a number of species including the rabbit (Zvaifler & Becker, 1966; Watanabe & Ovary, 1977). The antibody responsible for inducing PCA reactions in the rabbit has been identified as IgE and shows similar properties to human IgE (Zvaifler & Becker, 1966; Lindqvist, 1968; Revoltella & Ovary, 1969; Zvaifler & Robinson, 1969; Ishizaka *et al.*, 1970). In rabbits, IgE antibody can be raised by immunisation with a low dose (μ g quantities) of antigen mixed with aluminium hydroxide. This procedure produces maximum IgE titres after 120 days (Kravis & Zvaifler, 1974a) and a detailed study of the antibody was made by Stux & Ovary (1976). However, in this and other studies, no attempts were made to investigate which mediators were generated following antigen challenge, other than establishing that antihistamines were weak inhibitors.

In this paper, we have investigated the inflammatory response in the IgE-dependent PCA reaction in the rabbit to determine which mediators are released following antigen challenge. We have measured oedema formation in the PCA and used pharmacological agents to dissect the response. We also assessed the contribution of neutrophils to oedema formation. Apart from the involvement of vasodilator prostaglandins, none of the established mediators appear to contribute to inflammation in the PCA reaction suggesting the existence of novel mediators. We have also detected inflammatory activity in exudates obtained from passive anaphylactic reactions in the peritoneal cavity.

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Methods

Animals

Male New Zealand White specific pathogen-free rabbits (2.5–3.5 kg) were purchased from Froxfield Farm, Hampshire and Hacking and Churchill, Huntingdon, Cambridgeshire.

Generation of antiserum for PCA reactions

Antiserum for PCA reactions was raised in rabbits according to a schedule which stimulates preferential production of IgE antibodies (Kravis & Zvaifler, 1974a). The antigen (bovine gamma globulin (BGG), 1.5 µg per rabbit) was dissolved in saline, mixed with two volumes of aluminium hydroxide, washed twice and injected subcutaneously in a volume of 1 ml. The procedure was repeated three times at monthly intervals and after 4 months the rabbits were bled by carotid cannulation. Serum was prepared and those sera containing skin fixing anti-BGG antibodies (when tested at 1:10 dilution, see below), were pooled and passed through a column of Protein-A Sepharose to remove the major part of the IgG present. Aliquots of the IgG-depleted antiserum (referred to as anti-BGG IgE) were stored at –25°C. Non-immune rabbit serum depleted of IgG was used as a control for PCA reactions.

Generation of goat anti-rabbit C5a IgG

Rabbit C5a was purified, as the des Arg metabolite, from zymosan-activated serum by the successive use of batch-wise cation exchange, ethanol precipitation of unwanted proteins, gel filtration and cation-exchange high performance liquid chromatography (h.p.l.c.) as described previously (Williams & Jose, 1981; Jose *et al.*, 1983; Haslett *et al.*, 1989). A goat was immunized (20 × 0.1 ml i.m. injections in the hind quarters and 4 × 0.25 ml i.m. injections in the shoulder region) with the purified material (50 µg) emulsified in Freund's complete adjuvant on days 0 and 21. Boost injections in Freund's incomplete adjuvant were given every 21 days. Test bleeds taken 7–10 days after each booster injection showed similar antibody titres. The serum used in this paper was prepared from blood taken 10 days after the final antigen injection. For use as a neutralizing agent in the rabbit skin assay, the IgG fraction of the anti-serum was prepared by use of n-octanoic acid to precipitate unwanted proteins (Steinbuch & Audran, 1969). The IgG fraction was then precipitated with ammonium sulphate, resuspended in a volume equal to that of the original serum, dialysed against saline, filtered (0.2 µm) and stored in aliquots at –25°C. The antibody was shown to bind both C5a and C5a des Arg.

Radiolabelling of rabbit serum albumin

Preliminary studies revealed that the anti-BGG IgE used to sensitize skin sites for the PCA reaction recognised ¹²⁵I-labelled human serum albumin which is normally employed as the plasma marker in our studies. No cross-reactivity was found with rabbit serum albumin (RSA), therefore radioiodinated [¹²⁵I]-RSA was prepared according to the chloramine-T procedure (Hunter & Greenwood, 1962). Free iodine was removed with Sephadex-G25M. Sterile, pyrogen-free reagents and receptacles were used because the radiolabelled protein was for intravenous administration. The iodinated albumin (approx. 30 µCi mg⁻¹) was stored at 4°C in sterile saline containing 0.9% benzyl alcohol as preservative.

Neutrophil depletion

Rabbits were rendered neutropenic by a single i.v. injection of nitrogen mustard (freshly prepared in sterile saline) at a dose of 1.75 mg kg⁻¹ three days prior to the experiment

(Stetson, 1951; Wedmore & Williams, 1981). Under these conditions, the circulating neutrophil count three days later was approximately 1% of the pretreatment value.

For total and differential circulating leukocyte counts, samples of peripheral blood were taken from a central ear artery. Total leukocytes were determined in a Coulter counter (type ZBI) and May-Grunwald-Giemsa stain was used for differential counts on at least 200 cells.

Measurement of local oedema formation in rabbit skin

Oedema formation was measured as the local accumulation of intravenously injected [¹²⁵I]-RSA. Rabbits were anaesthetized with sodium pentobarbitone (30 mg kg⁻¹), the fur on the dorsal skin shaved and [¹²⁵I]-RSA (1.5 µCi kg⁻¹) mixed with a solution of Evans blue dye (10 mg kg⁻¹) in sterile saline was injected intravenously. Five minutes later agents under test, freshly prepared in sterile saline from stock solutions, were injected intradermally (0.1 ml volumes) in six replicates per treatment according to a balanced site injection plan. After 30 min, a 10 ml cardiac blood sample was taken into heparin for the preparation of plasma. The animal was then killed with an overdose of sodium pentobarbitone and the dorsal skin removed. Injection sites were punched out with a 17 mm diameter punch and counted together with three 1 ml plasma samples in a 12 head gamma counter (LKB, Wallac 1260 Multigamma). The amount of plasma protein that had accumulated in each skin site was calculated in terms of µl plasma by dividing the ¹²⁵I-count in each skin site by the ¹²⁵I-count in 1 µl plasma.

In the experiments designed to observe the effects of systemic administration of colchicine on oedema formation, the procedure was designed so that each animal served as its own control (Hellewell *et al.*, 1989). Test agents (antigen, FMLP + PGE₂ and bradykinin + PGE₂) were injected intradermally after i.v. administration of ¹²⁵I-RSA and Evans blue dye. Plasma protein accumulation was allowed to proceed for 30 min. Colchicine (1 mg kg⁻¹, or saline, 1 ml kg⁻¹, for the control; Hellewell *et al.*, 1989) was then injected intravenously and 5 min later the remaining skin sites were injected with the same combination of test agents. After a further 30 min, the animals were killed and local oedema formation was assessed as described above.

Passive cutaneous anaphylaxis (PCA)

Anti-BGG IgE (diluted 1:3, 1:10, 1:30 or 1:100 with sterile saline) was injected intradermally 48–72 h prior to antigen challenge. This was the optimum period required for IgE fixation (see Figure 2). After the fixation period, [¹²⁵I]-RSA was injected intravenously followed 5 min later by antigen challenge. BGG was administered either intravenously (5 mg kg⁻¹) or locally (1 µg 0.1 ml⁻¹) and local oedema formation was assessed as described above.

When drugs were tested intradermally, they were mixed with antigen or with combinations of inflammatory agents, prior to injection. One drug, L-659,989, was also tested intravenously as a 5 min pretreatment before the intradermal injection of test agents: these experiments were performed with pairs of animals, one rabbit receiving the drug, the other receiving vehicle.

Passive peritoneal anaphylaxis (PPA)

IgE-containing antiserum (2 ml, undiluted) or IgG-depleted non-immune serum (control) was injected into the peritoneal cavity of anaesthetized rabbits, followed by brief massage of the abdomen. After a 72 h fixation period, the animals were re-anaesthetized and 20 ml sterile saline was injected intraperitoneally followed by i.v. antigen challenge (5 mg kg⁻¹). At 90 min, the rabbits were killed with an anaesthetic overdose and the total available peritoneal exudate was collected into heparinized tubes on ice. After centrifugation (300 g) to

remove cells and debris, samples were analysed for inflammatory activity in the skin oedema assay using naive recipient rabbits. The time course of the peritoneal reaction was measured by removing 2 ml aliquots of exudate, at various times (30, 60, 120, 240 min) after antigen challenge, for testing in the skin oedema assay.

Drugs and materials

Arachidonic acid, colchicine, prostaglandin E_2 (PGE_2), bradykinin, indomethacin, N-formyl-methionyl-leucyl-phenylalanine (FMLP), phenylmethylsulphonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), rabbit serum albumin, zymosan and Freund's adjuvants were purchased from Sigma Chemical Co., Poole, Dorset; bovine gamma globulin (BGG), histamine and Evans blue dye were from British Drug Houses, Poole, Dorset; tissue kallikrein (Glumorin) and trasylol (Aprotinin) were from Bayer U.K. Ltd., Haywards Heath, West Sussex; PAF was from Bachem, Saffron Walden, Essex; aluminium hydroxide (4% in water; Aludrox) was from Wyeth Laboratories, Taplow, Berks; sodium pentobarbitone was from May and Baker, Dagenham, Essex; nitrogen mustard (Mustine HCl) and sterile 0.9% saline (Steriflex) was from Boots Company, Nottingham, Nottinghamshire; $Na^{125}I$ was from Amersham International, Amersham, Buckinghamshire; Protein-A Sepharose and Sephadex G-25M were from Pharmacia Fine Chemicals, Hounslow, Middlesex.

Zymosan-activated plasma (ZAP) was used as a source of C5a des Arg (approximately $3-5 \times 10^{-7}$ M). Briefly, rabbit heparinized (10 u ml^{-1}) plasma was incubated with zymosan (5 mg ml^{-1}) for 30 min at 37°C followed by centrifugation ($2 \times 10 \text{ min}$, 3000 g) to remove the zymosan. Aliquots of ZAP were stored at -25°C .

The following were obtained as gifts: mepyramine maleate was from May and Baker, Dagenham, Essex; cimetidine was from Smith Kline Beecham, Welwyn Garden City, Herts; L-659,989 (*trans*-2-(3-methoxy-5-methylsulphonyl-4-propoxy-phenyl)-5-(3,4,5-trimethoxy-phenyl) tetrahydrofuran) was from Dr J.C. Chabala, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey, USA; 48740 RP (3-(3-pyridyl)-1H,3H-pyrrolo [1,2-c] thiazole-7-carboxamide) was from Dr P. Sedivy, Rhone-Poulenc Sante, Vitry sur Seine, France; WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno(2,3-f)(1,2,4)-triazolo-(4,3-a) (1,4)-diazepine-2-yl]-1-(4-morpholinyl)-1-propanone) was from Dr H. Heuer, Boehringer Ingelheim KG, Ingelheim am Rhein, Federal Republic of Germany; REV 5901 (α -pentyl-3-(2-quinolinylmethoxy)-benzene-methanol) was from the Rorer Group Inc., Fort Washington, Pennsylvania, USA; B-3824, a synthetic bradykinin antagonist, was from Dr J.M. Stewart, University of Colorado School of Medicine, Denver, Colorado, USA.

Statistical analysis

Data are presented as the mean $\pm$ s.e.mean and have been analysed with Student's paired *t* test for within animal treatment, and with Student's unpaired *t* test for between animal treatments. A *P* value of <0.05 was considered statistically significant. Before calculating percentage inhibition of a response, the background value for i.d. injection of serum, saline or PGE_2 was subtracted from the data.

Results

Effect of heat treatment on IgE antibody

The effect of heating the anti-BGG IgE at 56°C for 30 min or 4 h on its ability to sensitize skin sites is shown in Figure 1. There was a heat-resistant component which is consistent with previous observations made with rabbit IgE (Revoltella

& Ovary, 1969; Kravis & Zvaifler, 1974a; Stux & Ovary, 1976).

Time course of IgE fixation in skin

In order to investigate the kinetics of the sensitization period, IgE-containing antiserum was injected intradermally 240, 144, 72, 48, 24, 6 h and 1 h before an intravenous injection of ^{125}I -albumin and antigen. Oedema formation was measured over 30 min. The results shown in Figure 2 demonstrate that

Figure 1 Effect of heat treatment of IgE-containing antiserum on its ability to sensitize skin for passive cutaneous anaphylactic (PCA) reactions. Undiluted antiserum was heated at 56°C for 30 min (●), or 4 h (□), or was untreated (○) before dilution with saline and injection into skin. After a fixation period of 72 h, antigen (BGG, 5 mg kg^{-1}) and ^{125}I -albumin were injected intravenously and oedema formation measured as the 30 min accumulation of ^{125}I -albumin in skin sites. The dashed line represents the background value obtained after i.d. injection of saline. Values shown are from 6 replicate injections in one rabbit.

Figure 2 The kinetics of sensitization of rabbit skin by IgE. Antiserum diluted 1:10 with saline was injected at different intervals before the intravenous injection of antigen (BGG, 5 mg kg^{-1}) and measurement of oedema formation. The dashed line represents the background value obtained after i.d. injection of saline. Values shown are from 6 replicate injections in one rabbit.

maximal oedema formation was obtained with a sensitization period of 48–72 h, although IgE persisted in skin for at least 10 days. Therefore, in all subsequent experiments a sensitization period of 48 or 72 h was used.

Duration of oedema formation in the PCA reaction

To establish the duration of oedema formation in the PCA following antigen challenge, the following experiment was performed. Antigen was injected locally into sensitized (72 h) skin sites at intervals before the i.v. injection of [^{125} I]-albumin at $t = 0$. Oedema formation was measured over a 30 min period. In this manner, PCA sites had been exposed to antigen for different time periods before the measurement of plasma leakage. As shown in Figure 3, oedema formation was short-lived, in this experiment a half-life of 15 min was obtained. In a total of three rabbits, the half-life value for oedema formation was 17 ± 3 min. We found no evidence for a late phase reaction in terms of oedema formation measured at 6 h post antigen challenge (data not shown). The latter experiments were performed in conscious rabbits, since anaesthesia had been demonstrated to interfere with late phase reactions in this species (Behrens *et al.*, 1987).

Effect of indomethacin on oedema formation in the PCA reaction

Figure 4 shows the effect of local administration of indomethacin on oedema formation in the PCA reaction. Indomethacin (10^{-8} mol), mixed with antigen prior to i.d. injection, reduced the PCA reaction by $41 \pm 3\%$ ($n = 7$ rabbits, $P < 0.002$). To confirm the cyclo-oxygenase inhibitory activity of indomethacin in the same group of rabbits, we examined the effect of the drug on oedema formation induced by i.d. injection of bradykinin mixed with arachidonic acid. The conversion of arachidonate to vasodilator prostaglandins in skin increases local blood flow and acts synergistically with the permeability-increasing activity of bradykinin to produce marked oedema formation (Williams & Peck, 1977). Indomethacin inhibited this response by $86 \pm 7\%$ ($P < 0.002$), i.e. reduced the response almost to that seen with bradykinin alone. Prostaglandin E_2 ($PG E_2$) and PGI_2 are potent vasodilators produced from arachidonate via the cyclo-oxygenase pathway, whereas none

Figure 3 The duration of oedema formation in the passive cutaneous anaphylactic (PCA) reaction. Skin sites sensitized 72 h previously with anti-BGG IgE (1:10 dilution) were injected with antigen (1 µg) at various time periods before the intravenous injection of [^{125} I]-albumin and measurement of oedema formation over a 30 min period. The interval between antigen challenge and albumin injection is shown on the abscissa scale. In this experiment the half-life for duration of oedema was approximately 15 min. The dashed line represents the background value obtained after i.d. injection of saline. Values shown are from 6 replicate injections in one rabbit and are representative of three experiments.

Figure 4 Effect of local administration of indomethacin on oedema formation in the passive cutaneous anaphylactic (PCA) reaction. Plasma leakage was induced by i.d. injection of BGG (1 µg) into PCA sites (1:3 dilution of IgE antiserum) and i.d. injection of bradykinin (BK; 10^{-10} mol) mixed with arachidonic acid (AA; 3×10^{-9} mol) into naive sites. Co-injection of indomethacin (10^{-8} mol) is indicated by the solid columns. The dashed line represents the background value obtained after i.d. injection of saline. Values shown are from 7 rabbits. * $P < 0.002$.

of known metabolites produced via this pathway increases microvascular permeability (Williams, 1979). Thus, it would appear that there is an endogenous vasodilator prostaglandin component in the PCA reaction, although it is likely that other vasodilators are also involved.

Effect of antihistamines on oedema formation in the PCA reaction

Figure 5 shows the effects of local administration of H_1 (mepyramine) and H_2 (cimetidine) antihistaminic drugs, alone and in combination, on the PCA reaction. Mepyramine (3×10^{-9} mol) inhibited the leakage response to i.d. injection of histamine mixed with $PG E_2$ by $92 \pm 3\%$ ($P < 0.01$, $n = 6$ rabbits). Cimetidine (3×10^{-7} mol) produced a partial but significant ($P < 0.02$) suppression of the response to histamine + $PG E_2$ ($42 \pm 7\%$ inhibition) and did not further reduce the inhibition seen with mepyramine alone ($94 \pm 2\%$). Local administration of mepyramine in the PCA produced no significant suppression of the leakage response: $13 \pm 5\%$ inhibition ($P > 0.05$) and $20 \pm 8\%$ inhibition ($P > 0.05$) at 1:3 and 1:10 dilutions of the anti-BGG IgE respectively. Cimetidine alone produced no inhibition of the PCA, although when combined with mepyramine did produce significant inhibition ($27 \pm 8\%$ inhibition, $P < 0.05$) of the response at 1:10 dilution of the sensitizing antibody. Thus histamine appears to play a minor role in mediating oedema formation in the PCA reaction.

Effect of PAF antagonists on the PCA reaction

The effect of local administration of two potent PAF antagonists, WEB 2086 (Casals-Stenzel *et al.*, 1987) and L-659,989 (Ponpipom *et al.*, 1988), on oedema formation in the PCA reaction is shown in Figure 6a. WEB 2086 (10^{-7} mol) and L-659,989 (5×10^{-8} mol) inhibited the leakage response to i.d. injection of PAF + $PG E_2$ by $94 \pm 2\%$ and $88 \pm 5\%$ respectively ($n = 4$ rabbits) in concordance with our earlier published data (Hellewell & Williams, 1989a). In the same group of animals, neither antagonist significantly affected oedema formation in the PCA reaction (Figure 6a). We also tested another selective, but less potent PAF antagonist, 48740 RP (Sedivy *et al.*, 1985; Hellewell & Williams, 1989a). 48740 RP (10^{-6} mol) inhibited the oedema response to PAF-

Figure 5 Effect of local administration of antihistamines on oedema formation in the passive cutaneous anaphylactic (PCA) reaction. Antigen ($1 \mu\text{g}$ BGG) was injected into sensitized skin sites (1:10 and 1:3 dilutions of antiserum) either alone (open columns) or mixed with mepyramine (3×10^{-9} mol, solid columns), cimetidine (3×10^{-7} mol, hatched columns) or a combination of both drugs (stippled columns). Histamine (10^{-8} mol) was mixed with prostaglandin E_2 (PGE_2 , 3×10^{-10} mol) and the antagonists before injection. The dashed lines represent the control values obtained after i.d. injection of BGG into sites pretreated with a 1:3 dilution of non-immune serum (control for PCA) or after i.d. injection of saline (control for histamine + PGE_2). Values shown are from 6 rabbits. * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$.

+ PGE_2 by $60 \pm 5\%$ ($n = 5$ rabbits), while the PCA reaction (1:10 dilution of sensitizing antibody) was unaffected: leakage in the PCA was $38 \pm 5 \mu\text{l}$ and in the presence of 48740 RP was $41 \pm 2 \mu\text{l}$.

In a further series of experiments we investigated the effect of i.v. administration of L-659,989 (5 mg kg^{-1}) on oedema formation in the PCA reaction. The results are shown in Figure 6b. Oedema formation induced by PAF + PGE_2 was inhibited by $88 \pm 8\%$ ($P < 0.005$), $n = 4$ pairs of rabbits. However, oedema formation in the PCA reaction was unaffected by the PAF antagonist. Weaker PCA responses (1:30 and 1:100 dilution of the sensitizing antibody were also unaffected by i.v. L-659,989 (data not shown). Thus, PAF does not appear to contribute to oedema formation in the PCA reaction.

Effect of trasylol and a bradykinin antagonist on the PCA reaction

The possibility of kinin formation in skin was investigated by use of trasylol (a kallikrein inhibitor) and a bradykinin antagonist, B-3824 (Vavrek & Stewart, 1985). Local administration of trasylol (1.5×10^{-9} mol) inhibited oedema induced by kallikrein (500 ng) + PGE_2 (3×10^{-10} mol) by $98 \pm 2\%$ ($n = 3$ rabbits). In the same animals, at a 1:10 dilution of sensitizing antibody, leakage in the PCA was $35 \pm 1 \mu\text{l}$ and in the presence of trasylol was $38 \pm 6 \mu\text{l}$; at a 1:3 dilution of antibody the values were $65 \pm 13 \mu\text{l}$ and $72 \pm 19 \mu\text{l}$, respectively (μl per skin site subtracted for values in the absence of antigen). Preliminary studies showed that plasma leakage in the PCA was also unaffected by local administration of SBTI (1 mg), PMSF (6×10^{-8} mol) or EDTA (10^{-6} mol).

In two rabbits, local administration of the antagonist (10^{-8} mol) reduced oedema formation induced by a mixture of bradykinin (10^{-10} mol) and PGE_2 (3×10^{-10} mol) by 94% and 88% respectively. Oedema formation in the PCA was not inhibited by B-3824. In fact, responses in both animals showed some increase, e.g. in one animal, oedema at 1:10 and 1:3 dilution of the antibody was $39 \pm 6 \mu\text{l}$ and $82 \pm 10 \mu\text{l}$ in antigen-injected sites, respectively, compared with

Figure 6 Effect of (a) local or (b) intravenous administration of PAF antagonists on oedema formation in the passive cutaneous anaphylactic (PCA) reaction. (a) Antigen ($1 \mu\text{g}$ BGG) was injected into sensitized skin sites (1:10 and 1:3 dilutions of antiserum) either alone (open columns) or mixed with WEB 2086 (10^{-7} mol, solid columns) or L-659,989 (5×10^{-8} mol, hatched columns). PAF (10^{-9} mol) was mixed with prostaglandin E_2 (PGE_2 , 3×10^{-10} mol) and the antagonists before injection. (b) Skin sites were sensitized with a 1:10 dilution of antiserum. Rabbits were treated with dimethylformamide as controls (0.1 ml kg^{-1} i.v., open columns) or with L-659,989 (5 mg kg^{-1} i.v., hatched columns). After 5 min, antigen was injected intravenously (BGG, 5 mg kg^{-1}). PAF (10^{-9} mol) was mixed with PGE_2 (3×10^{-10} mol) prior to injection. The dashed lines represent the background values obtained after i.d. injection of BGG into sites pretreated with a 1:3 dilution of non-immune serum or after i.d. injection of saline. Values shown are from 4 rabbits in (a) and from 4 pairs of rabbits in (b). * $P < 0.005$.

$63 \pm 9 \mu\text{l}$ ($P < 0.05$) and $107 \pm 5 \mu\text{l}$ ($P < 0.05$) in sites injected with antigen plus B-3824. We attributed the increase to partial agonist activity of B-3824.

Effect of a 5-lipoxygenase inhibitor on oedema formation in the PCA reaction

The effect of REV-5901, a selective 5-lipoxygenase inhibitor with little anti-oxidant properties, is shown in Figure 7. REV-5901, at a dose (10^{-7} mol) which has previously been shown to inhibit 5-lipoxygenase in rabbit skin (Aked *et al.*, 1986), had no significant inhibitory effect on oedema formation in the PCA reaction. The 5-lipoxygenase inhibitory activity of the drug was evaluated in the same experiments by injecting it with a high dose of arachidonic acid (10^{-7} mol), since the leakage response to the latter is dependent on leukotriene B_4 (LTB_4) generation in rabbit skin (Aked & Foster, 1987). Oedema induced by arachidonate was inhibited by $49 \pm 7\%$ ($P < 0.02$), demonstrating that 5-lipoxygenase activity in skin was reduced in these experiments.

Figure 7 Effect of local administration of REV 5901 on oedema formation in the passive cutaneous anaphylactic (PCA) reaction. Plasma leakage was induced by injection of antigen (BGG, 1 µg) into PCA sites (1:3 dilution of the sensitizing antibody) and i.d. injection of arachidonic acid (AA, 3×10^{-7} mol). The co-injection of REV 5901 (10^{-7} mol) is indicated by the solid columns. The dashed line represents the background value obtained after i.d. injection of saline. Values shown are from 6 rabbits. * $P < 0.02$.

Effect of anti-C5a antibodies on the PCA reaction

We have previously used anti-C5a antibodies to inhibit selectively C5a-induced inflammation in rabbit skin (Jose & Williams, 1987; Hellewell *et al.*, 1988; Collins *et al.*, 1991). Therefore, we tested the effects of these antibodies on the PCA reaction. Anti-C5a IgG, but not IgG prepared from non-immune serum, abolished oedema formation induced by C5a des Arg (ZAP) mixed with PGE₂; leakage was reduced from 35 ± 4 µl to 2 ± 2 µl (µl per skin site subtracted for values obtained after i.d. injection of PGE₂, mean $\pm$ s.e.mean for 6 replicate injections). In contrast, the inflammatory response induced by FMLP (5×10^{-11} mol) + PGE₂ was unaffected by anti-C5a (control, 53 ± 7 µl; + anti-C5a, 64 ± 4 µl) as was the PCA reaction (control, 48 ± 8 µl; + anti-C5a, 49 ± 6 µl). Thus, endogenous C5a does not appear to be involved in the PCA. Similar data were found in a second rabbit.

Role of circulating neutrophils

We have previously used nitrogen mustard and colchicine to distinguish between mediators which induce oedema formation via a neutrophil-endothelial interaction or via a direct effect on the microvascular endothelium (Wedmore & Williams, 1981; Hellewell *et al.*, 1989). Figures 8 and 9 show the effects of these anti-neutrophil treatments on oedema formation in the PCA reaction. For comparison, C5a (ZAP) and FMLP were used as standard neutrophil-dependent mediators and bradykinin as a standard neutrophil-independent mediator. In these experiments, the small contribution of the histamine was blocked by the intravenous injection of mepyramine (3 mg kg^{-1}).

Figure 8 shows the results from experiments conducted in control and nitrogen mustard-treated (neutropenic) rabbits. The circulating neutrophil counts at the time of antigen challenge were $2.1 \pm 0.3 \times 10^6 \text{ ml}^{-1}$ and $2.5 \pm 0.6 \times 10^4 \text{ ml}^{-1}$ respectively. Oedema formation induced by ZAP + PGE₂ was virtually abolished in neutropenic rabbits: responses obtained with undiluted and 1:10 dilutions of ZAP were inhibited by $85 \pm 3\%$ and $81 \pm 6\%$ respectively. In contrast, the responses to bradykinin + PGE₂ were unaffected by neutrophil depletion. In the PCA reaction, leakage responses at 1:100 and 1:30 dilutions were not significantly affected in neutropenic rabbits, however there was a significant inhibition ($43 \pm 10\%$, $P < 0.05$) at 1:10 dilution of the sensitizing antibody (Figure 8).

The effects of colchicine on the PCA reaction are shown in

Figure 8 Effect of neutrophil depletion on oedema formation in the passive cutaneous anaphylactic (PCA) reaction. Responses were obtained in control rabbits (open symbols) and in rabbits treated 3 days previously with nitrogen mustard (1.75 mg kg^{-1} , i.v., closed symbols). C5a (ZAP, undiluted and diluted 1:10) and bradykinin (BK, 10^{-11} and 10^{-10} mol) were mixed with prostaglandin E₂ (PGE₂, 3×10^{-10} mol) prior to injection. PCA reactions were with 1:100, 1:30 and 1:10 dilutions of the sensitizing antibody given intradermally 48–72 h before the intravenous injection of antigen (BGG, 5 mg kg^{-1} , i.v.). All rabbits were pretreated with mepyramine (3 mg kg^{-1} , i.v.). The dashed lines represent the background value obtained after i.d. injection of saline. Values shown are from 6 pairs of rabbits. * $P < 0.05$; ** $P < 0.001$.

Figure 9. Figure 9a shows that i.v. administration of saline had no significant effect on oedema responses to FMLP, bradykinin or the PCA (i.e. responses over 35–65 min were the same as those measured over 0–65 min). In contrast, Figure 9b shows that systemic treatment with colchicine produced a dramatic decrease in FMLP-induced oedema formation without significantly affecting that induced by bradykinin. Responses to 5×10^{-11} and 5×10^{-12} mol FMLP were inhibited by $84 \pm 5\%$ ($P < 0.002$) and $82 \pm 6\%$ ($P < 0.002$), while responses to 10^{-10} and 10^{-11} mol bradykinin were not significantly reduced. Colchicine inhibited oedema formation in the PCA reaction: the inhibition at 1:10 dilution of the sensitizing antibody ($45 \pm 8\%$, $P < 0.05$) was similar to that seen with nitrogen mustard. At 1:3 dilution of antibody, the colchicine-dependent component was more marked; $54 \pm 9\%$ inhibition, $P < 0.02$.

Taken together, the results in Figures 8 and 9 suggest that there is a neutrophil-dependent component to oedema formation in the PCA reaction although this appears to be proportional to the magnitude of the reaction.

Generation of permeability-increasing activity in passive peritoneal anaphylaxis (PPA)

In an attempt to isolate and thus characterize inflammatory mediators in anaphylactic reactions, we carried out experiments in the peritoneal cavity. Exudate was obtained from the cavity and mixed with PGE₂ before injection in the skin of other rabbits. Figure 10a shows the time course of generation, following antigen challenge, of permeability-increasing activity in peritoneal anaphylactic exudate. Maximal activity was obtained 1–2 h after antigen challenge and had virtually disappeared at 4 h. A small amount of activity was also detected in the control exudate collected 30 min after antigen challenge of non-sensitized animals.

Figure 10b shows the results of further experiments in which exudate fluids, collected 90 min after antigen challenge in sensitized and control rabbits, were tested for inflammatory activity in the skin assay. Anaphylactic exudate

Figure 9 Effect of colchicine on oedema formation in the passive cutaneous anaphylactic (PCA) reaction. Reactions were initiated by the local injection of antigen (BGG, 1 µg) 48–72 h after the intradermal injection of sensitizing antibody. For comparison, oedema responses were induced by i.d. injection of FMLP + prostaglandin E₂ (PGE₂, 3 × 10⁻¹⁰ mol) and bradykinin (BK) + PGE₂. All rabbits were pretreated with mepyramine (3 mg kg⁻¹ i.v.). Panel (a) shows responses before (○) and after (●) i.v. saline (1 ml kg⁻¹) and panel (b) shows responses before (○) and after (●) i.v. colchicine (1 mg kg⁻¹). The dashed lines represent the background value obtained after i.d. injection of saline. Values shown are from 6 pairs of rabbits.

*P < 0.05, **P < 0.02, ***P < 0.002.

showed inflammatory activity which was potentiated by the addition of PGE₂. The control exudates displayed some inflammatory activity in the skin but this was much weaker than the response to anaphylactic exudates.

The generation of inflammatory activity in the peritoneal anaphylactic exudates allows the possibility of characterizing and purifying inflammatory mediators of anaphylactic reactions in the rabbit.

Discussion

In this study we investigated which inflammatory mediators were responsible for the local oedema formation observed in the rabbit PCA reaction. In rabbit skin it has been established that oedema formation is dependent on a synergism

Figure 10 Generation of permeability-increasing activity in a passive peritoneal anaphylactic (PPA) reaction. Kinetics of the generation are shown in (a). Peritoneal exudate was removed at the intervals shown after intravenous antigen challenge (BGG, 5 mg kg⁻¹), mixed with prostaglandin E₂ (PGE₂ 3 × 10⁻¹⁰ mol), injected i.d. into a naive rabbit and oedema formation assessed after 30 min (●). The control (○) represents exudate from an animal that received non-immune serum i.p. (see Methods for details). The effect of PGE₂ on oedema formation induced by control and PPA 90 min exudates is shown in (b). Exudate, removed 90 min after antigen challenge to control and sensitized animals was injected alone (open columns), or mixed with PGE₂ (3 × 10⁻¹⁰ mol) (solid columns). The dashed lines represent the background values obtained after i.d. injection of saline. Values shown in (a) are from 6 replicate injections per time point and those shown in (b) are from 5 control exudates and 7 PPA exudates.

between mediators which increase local blood flow and those which increase vascular permeability (Williams & Morley, 1973; Williams & Peck, 1977). Blocking the generation or action of either type of mediator results in a decrease in local oedema formation.

Experiments with indomethacin established that a vasodilator prostaglandin was generated in the PCA reaction although, since the reaction was not abrogated by this treatment, it is likely that other vasodilators are also generated. Possibilities include neuropeptides and we are currently investigating this. Vasodilator prostaglandins are known to be generated in other cutaneous reactions in the rabbit including those in response to zymosan (Wedmore & Williams, 1981), polycations (Needham *et al.*, 1988) and in the reversed passive Arthus reaction (Williams *et al.*, 1986).

Based on the weak inhibitory actions of a range of potent and selective pharmacological agents it appears that none of the established mediators is involved in increasing vascular permeability. The small inhibitory effect of antihistamines is consistent with earlier studies of the rabbit PCA reaction

(Lindqvist, 1968; Zvaifler & Robinson, 1969; Zvaifler *et al.*, 1971). This is in contrast to PCA reactions in the monkey where a combination of H_1 and H_2 antihistamines completely inhibit the wheal observed after antigen challenge (Hutchcroft *et al.*, 1979) and in man in which a combination of antihistamines reduced by approximately 50% the wheal response after antigen (Smith *et al.*, 1980). However, there is evidence that these observed effects are due not only to histamine antagonism but to an additional mast cell stabilizing effect (Dorsch *et al.*, 1982). Histamine has also been detected in blister fluid of skin sites challenged with antigen in sensitized individuals (Pienkowski *et al.*, 1988) and, in the rabbit PCA, the histamine content of sensitized skin sites was reduced substantially after antigen challenge (Zvaifler *et al.*, 1971). 5-Hydroxytryptamine (5-HT) up to 10^{-7} mol/site, has no permeability-increasing activity in rabbit skin (Hellewell, unpublished) and was therefore discounted as a mediator in the PCA; this is in contrast to its potent permeability-increasing effects in rodents. In addition, earlier studies of Zvaifler *et al.*, (1971) found no inhibitory action of methysergide, alone or in combination with an H_1 antihistamine, on bluing in the rabbit PCA.

PAF has been proposed as an important mediator of IgE-mediated systemic anaphylaxis in the rabbit (Halonen *et al.*, 1980). However, a recent study using the PAF antagonist WEB 2086 concluded that PAF plays a relatively minor role in respiratory, circulatory and haematological alterations that occur in anaphylaxis, although there was a significant reduction in total pulmonary resistance (Lohman & Halonen, 1990). These observations indicate that other mediators are likely to be involved in mediating the anaphylactic alterations. In man, the PAF antagonist BN-52063 has been shown to reduce significantly wheal volume in the cutaneous late phase response (at 8 h) in response to local antigen challenge in sensitized individuals (Roberts *et al.*, 1988). However, although the immediate response was reduced by 50%, this was not a significant effect (Roberts *et al.*, 1988). In our studies, administration of three PAF antagonists locally and one intravenously had no significant effects on local oedema formation in the PCA suggesting that endogenous PAF was unlikely to be involved in increasing vascular permeability.

There was no evidence for the generation of 5-lipoxygenase products since REV-5901 was without effect. As LTC_4 and LTD_4 are weak inducers of permeability changes when injected intradermally in rabbit skin (Ueno *et al.*, 1981), this suggests that LTB_4 , which does increase permeability (Wedmore & Williams, 1981), was not involved in the PCA reaction. A bradykinin antagonist, B-3824, and a kallikrein inhibitor, trasylol, were also without effect.

The contribution of circulating neutrophils to local oedema formation was supported by two different methods; neutrophil depletion using nitrogen mustard and interference of neutrophil function with colchicine. Both methods have been used to demonstrate neutrophil-dependent oedema formation (Wedmore & Williams, 1981; Hellewell & Williams, 1989) and Zvaifler *et al.* (1971) found that nitrogen mustard treatment caused very effective inhibition of bluing in the rabbit PCA reaction when combined with mepyramine. In the present study, mepyramine was used to inhibit the small contribution of histamine to oedema formation. Thus, it appeared that there was a neutrophil-dependent mediator in the rabbit PCA reaction that induced plasma exudation and that the larger the PCA reaction in terms of oedema formation, then the greater dependence on circulating neutrophils. Possibilities included C5a and C5a des Arg (Wedmore & Williams, 1981), tumour necrosis factor- α (TNF- α) (Rampart *et al.*, 1989a), interleukin-1 (IL-1) (Buckley *et al.*, 1991) and IL-8 (Rampart *et al.*, 1989b). PAF (Hellewell, 1990) and LTB_4 (Wedmore & Williams, 1981) were already discounted (see above).

The likelihood that C5a was involved was small since the IgE antibody would not be expected to be complement fixing. Henson & Cochrane (1969) and Stux & Ovary (1976)

both reported an anaphylactic IgG antibody in the rabbit which induced a PCA reaction that was dependent on an intact complement system. However, we could not find evidence for the existence of this antibody in our experiments. It was possible that cell-derived proteases, released in the PCA reaction, could cleave C5 in tissue fluid leading to the generation of C5a (Orr *et al.*, 1979). Nevertheless, the contribution of C5a was mitigated by the observation that antibodies to C5a, which abrogated local oedema and neutrophil responses to i.d. ZAP and had no effect on the responses to FMLP, were without effect on oedema formation in the PCA reaction. In other studies, the same antibody effectively reduced plasma leakage and neutrophil accumulation in the reversed passive Arthus reaction in rabbit skin (Hellewell *et al.*, 1988).

Human mast cells are a source of preformed tumour necrosis factor- α (TNF- α) (Gordon & Galli, 1990) which can be released rapidly. As TNF- α induces neutrophil accumulation and neutrophil-dependent plasma exudation in rabbit skin (Rampart *et al.*, 1989a), its role in the PCA reaction deserves investigation. Both IL-1 and IL-8 induce neutrophil-dependent plasma leakage in rabbit skin (Buckley *et al.*, 1991; Rampart *et al.*, 1989b) however, there is no evidence that either cytokine is stored in cells; rather, their release depends on gene induction and protein synthesis, which takes 1–2 h to occur (Arai *et al.*, 1990; Oppenheim *et al.*, 1991). Nevertheless, it would still be valuable to assess their role using reagents such as blocking antibodies.

Earlier studies of the rabbit PCA focussed on the effects of antihistamines which produced varying degrees of inhibition, albeit small. Zvaifler *et al.* (1971) studied additional compounds which also had little effect. These included diethylcarbamazine (now known to be a non-specific 5-lipoxygenase inhibitor), disodium chromoglycate (to inhibit mast cell degranulation) and cobra venom factor (to reduce the blood complement titre). Kravis & Zvaifler (1974b) found that infusions of aminophylline, isoprenaline and propranolol were also without effect but did find reductions in local oedema formation (as assessed by diameter of bluing) after systemic treatment with PGE_1 and histamine. However, these latter treatments were associated with profound hypotension, an effect which is known to attenuate cutaneous oedema responses (Rampart & Williams, 1986). Thus, these earlier studies provide few clues as to the permeability-inducing agents that are generated in the rabbit PCA.

It is not known which cell type in skin was sensitized by the IgE antibody. Bauer *et al.* (1972) identified mast cells in rabbit skin and showed that these disappeared following antigen challenge in PCA sites. Furthermore, anti-IgE antibodies induce oedema formation in rabbit skin (Hellewell & Williams, 1989b) although it is not known if this is a consequence of cross-linking IgE already fixed on mast cells. In other species, including man, it is assumed that IgE-mediated cutaneous anaphylactic responses are the result of IgE-dependent mast cell degranulation (Dolovich *et al.*, 1973; Solley *et al.*, 1976; Dor *et al.*, 1983). In addition, late phase responses (LPR) characterized by subcutaneous oedema and erythema (Dolovich *et al.*, 1973; Solley *et al.*, 1976) are also found in some individuals. We found no evidence for a late leakage response in the PCA; in fact the response was almost complete by 60 min. Moreover, we did not detect a late (up to 6 h) increase in local blood flow as assessed by a lack of potentiation of oedema when bradykinin was injected into PCA sites after a delay (data not shown).

In an attempt to isolate oedema-inducing activity in tissue fluid, we generated IgE-mediated anaphylactic reactions in the peritoneal cavity. Following antigen challenge there was an increase in permeability-increasing activity above the control exudate, as measured using the skin as an *in vivo* bioassay. This was detected within 30 min of antigen challenge and peaked after 90–120 min. Preliminary experiments confirmed that the activity was not reduced in the presence of antihistamines and was unstable to freezing. Currently, we

are attempting to isolate the activity. Studies in the guinea-pig have shown that histamine, PAF and a 5-lipoxygenase product are involved in local oedema formation in the PCA reaction (Weg *et al.*, 1991) and in allergic human subjects, skin responses to local antigen injection have been shown to involve histamine (Smith *et al.*, 1980) with evidence for the generation of PGD₂ (Pienkowski *et al.*, 1988), LTC₄ and LTD₄ (Talbot *et al.*, 1985) and interleukin-1 (Bochner *et al.*, 1990).

In conclusion, we have characterized local oedema forma-

tion in the IgE-dependent rabbit PCA reaction and found evidence for the involvement of a vasodilator prostaglandin and unknown mediators of increased vascular permeability. Current studies are attempting to identify this activity or activities which may have relevance for IgE-dependent human allergic diseases.

We thank the National Asthma Campaign and Pfizer Central Research, U.S.A. for support.

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(Received June 15, 1992)

Revised August 13, 1992

Accepted August 17, 1992)