MEDIATION OF INCREASED VASCULAR PERMEABILITY AFTER COMPLEMENT ACTIVATION

Histamine-independent Action of Rabbit C5a*

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Complement activation in rabbit plasma has been found to result in the generation of a factor which is able to increase microvascular permeability by a mechanism independent of histamine release. Evidence has been obtained that this factor corresponds with the major chemotactic factor (1) in complement-activated rabbit plasma, C5a, probably devoid of its carboxyl terminal arginine (2, 3). This substance was found to produce little plasma exudation when injected alone into skin. However, when mixed with a vasodilator prostaglandin (PG) (PGE_1, PGE_2, or PGI_2),[1] C5a was found to be more potent at inducing plasma exudation than either of the established permeability-increasing agents, histamine or bradykinin. This interdependence of two types of mediator, a vasodilator (with no action on permeability) and a permeability-increasing substance (with no vasodilator activity) may explain why rabbit C5a has not previously been associated with increased vessel wall permeability. Generally, complement activation is thought to lead to increased vascular permeability because of histamine released from mast cells and basophils by the anaphylatoxins C3a and C5a (4–6). The finding that C5a can increase vascular permeability by an action independent of histamine release, considerably extends the potential importance of C5a in inflammatory edema because, in this respect, histamine has a very limited role in most clinical and experimental situations.

It is proposed that C5a, although discovered originally because of its systemic effects from which its trivial name anaphylatoxin is derived (7), has evolved as a local hormone whose extravascular generation can mediate the local accumulation of fluid and leukocytes. In this way, C5a can control local complement-mediated lysis and phagocytosis of microorganisms in tissues. Both local fluid accumulation and leukocyte accumulation in response to C5a suggest an intimate relationship between this substance and vascular endothelial cells.

Preliminary results of this investigation have been described previously (8, 9).

Materials and Methods

Animals. Male New Zealand white rabbits, 3.5–4 kg body wt, were used in all experiments.

Measurement of Plasma Exudation in Rabbit Skin. Plasma exudation in skin was measured as the

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CM, carboxymethyl; ED_{50}, mean effective dose; OA, ovalbumin; PAPA, plasma activation permeability agent; PG, prostaglandin; PMN, polymorphonuclear; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; ThG, thyroglobulin.

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accumulation of intravenously injected ^{131}I-human serum albumin, as previously described (10, 11). ~15 μCi/kg body wt of ^{131}I-human serum albumin, mixed with 2 ml of 2.5% wt:vol Evans blue dye in 0.9% saline, was injected via the marginal ear vein of the rabbit. This was followed by an intravenous injection of a short acting anesthetic, methohexitone sodium (~10 mg/kg). Intradermal injections (0.1-ml vol) of test materials were then administered into the previously clipped back skin. Injections were given in random order according to a balanced site pattern. Up to 14 test materials, each having 6 replicates, were injected into each rabbit. After 30 min the animal was killed by an overdose of pentobarbitone sodium, and 1-ml blood samples were taken three times by cardiac puncture. The back skin was then removed and the injection sites punched out using a 16-mm diameter steel punch. The skin and blood samples were then counted in an automatic gamma spectrometer. The skin sample counts were divided by the count of 1 μl of blood plasma so that exudate volumes could be expressed in terms of microliters of plasma (11).

The time-course of the response to intradermally injected zymosan was measured by injecting zymosan at different intervals before the intravenous injection of labeled albumin; exudation was then measured over a period of 30 min. The effect of vasodilator PG on these responses was determined by injecting the PG (100 ng/0.1 ml injection) into the skin sites immediately after intravenous injection of labeled albumin; the PG routinely used in these experiments was PGE_1. Pharmacological inhibitors or antagonists were injected locally.

Measurement of Changes in Blood Flow in Rabbit Skin. Changes in blood flow were measured in rabbit skin using a multiple site radioactive xenon clearance technique (10, 11). Test samples were mixed with solutions of ^{133}Xe to give an activity of 5–10 μCi/0.1 ml and injected intradermally as above. Animals were killed after a period of 20 min, the skin was removed and the injection sites punched out. Skin samples and samples of injection fluids were stored under paraffin oil in sealed tubes for counting. Results, expressed as percentage increased or decreased blood flow with respect to saline-injected control sites, were calculated as previously described (11).

Activation of Plasma. Small volumes of blood (up to 30 ml) were collected into heparin (10 U/ml) from the marginal ear veins of conscious rabbits. Larger volumes were obtained from the cannulated carotid artery of anesthetized rabbits (pentobarbitone sodium, ~30 mg/kg). Blood was centrifuged for 25 min at 1,900 *g* and the plasma removed. Zymosan was suspended in 0.9% saline at a concentration of 10 mg/ml (except where otherwise stated in the text), and 0.1 ml of this suspension was added to every 1 ml of plasma. Plasma was incubated at 37°C for 30 min in a shaking water bath. Control plasma was incubated in the same way with 0.1 ml of saline per 1 ml of plasma. Test and control plasma samples were centrifuged for 20 min at 1,900 *g*. The supernates were removed and tested immediately, fractionated at 2°C, or stored at −20°C.

Fractionation of Plasma. All steps were carried out at 2°C. Use of glassware was avoided except for chromatography columns which were siliconized before use.

Plasma was acidified to pH 5.5–6.0 by slowly adding 0.2 M sodium acetate buffer, pH 3.8, centrifuged, and applied to columns (1.5 × 2 cm) of carboxymethyl (CM)-Sephadex C-25 equilibrated in 0.9% saline:0.1 M sodium acetate buffer pH 6.0 (5:1). At least 100 ml of activated plasma could be passed through a single column without any permeability-increasing activity emerging in the effluent. The columns were washed with 40 ml saline:sodium acetate buffer pH 6.0, and the permeability-increasing factor was then eluted with 30 ml 0.5 M ammonium formate. The lyophilized ammonium formate fraction was chromatographed in an upward direction on columns of Sephadex gel filtration media. For analytical purposes, a column (1.5 × 87 cm) of Sephadex G-75 superfine in 0.9% saline was used. For preparative procedures, a column (2.5 × 70 cm) of Sephadex G-100 in 0.9% saline:0.2 M sodium acetate buffer, pH 3.8 (9:1), was used; fractions with permeability-increasing activity (or equivalent fractions from plasma incubated in the absence of zymosan) were pooled and lyophilized. Excess salt was removed using a column (1.5 × 5 cm) of Sephadex G-25 M in saline:sodium acetate buffer pH 3.8, and preparations were stored at −20°C. For biological testing, portions were removed, neutralized with sodium hydroxide, and diluted with 0.9% saline:0.2 M sodium phosphate buffer pH 7.2 (9:1). Protein concentrations were determined using a turbidometric tannin micromethod (12). In some experiments (as indicated in the text), the ion-exchange step

was omitted, and whole plasma was chromatographed on columns of Sephadex G-100 (2.6 × 65 cm) in 0.9% saline.

Columns of Sephadex gels were calibrated using the following proteins with molecular weights (13) as indicated: bovine serum albumin (BSA, 67,000); ovalbumin (OA, 45,000); soybean trypsin inhibitor (SBTI, 21,500); bovine pancreas ribonuclease A (RNase, 13,700); and glucagon (3,500). The void volume was determined using bovine thyroglobulin (ThG, 670,000) and the salt peak with ammonium formate.

Materials. Mepyramine maleate and pentobarbitone sodium were purchased from May and Baker Ltd., Dagenham, Essex, United Kingdom; methohexitone sodium from Eli Lilly & Co., Windlesham, Surrey, United Kingdom; heparin sodium from Duncan, Flockhart & Co., London, United Kingdom; Evans Blue dye from Searle Diagnostics, High Wycombe, Buckinghamshire, United Kingdom; histamine acid phosphate from British Drug Houses, Ltd., Poole, Dorset, United Kingdom; Trasylol (aprotonin) from Bayer UK Limited, Pharmaceutical Division, Haywards Heath, West Sussex, United Kingdom; bradykinin triacetate, norepinephrine bitartrate, prostaglandins E_1 and E_2, SBTI, zymosan (zymosan A, from *S. cerevisiae*), and proteins for calibrating gel filtration columns from Sigma Chemical Co., Poole, Dorset, United Kingdom; Sephadex gel filtration and ion exchange materials from Pharmacia Fine Chemicals, Hounslow, Middlesex, United Kingdom; ^{131}I-human serum albumin and ^{133}Xe in saline from The Radiochemical Centre, Amersham, Buckinghamshire, United Kingdom.

The following were gifts: hog C5a from Professor W. Vogt, Max Planck Institut, Göttingen, German Federal Republic; glucagon from Dr. W. Dawson, Eli Lilly & Co.; mammalian tissue kallikrein (Glumorin) from Bayer; and indomethacin from Merck, Sharp & Dohme, Hoddesdon, Hertfordshire, United Kingdom.

Results

Involvement of Endogenous PG in Plasma Exudation Induced by Intradermal Injections of Zymosan. Fig. 1 shows the time-course of the rate of plasma exudation after intradermal injection of zymosan in the rabbit. Zymosan was injected intradermally at intervals before an intravenous injection of ^{131}I-albumin, and plasma exudation was then measured over periods of 30 min as shown on the abscissa. Little plasma exudation was apparent during the first 30 min. Peak exudation rate was between 1 and 3 h; exudation then declined reaching negligible rates by 5 h. Local injection of the PG synthesis inhibitor, indomethacin, effectively suppressed plasma exudation, as shown. PGE_1 (PGE_2 and PGI_2 showed similar activity) potentiated exudation when injected locally immediately after intravenous injection of labeled albumin. Interestingly, potentiation was elicited by PG during the first 30 min, at a time when zymosan alone produced negligible exudation. PG injected alone produced little plasma exudation, as shown. In spite of the observation that indomethacin suppressed the response to zymosan, indomethacin had no effect on the zymosan response when potentiated by PGE_1.

These observations can be explained in the following manner. Zymosan stimulates the generation of two substances in the skin, neither of which induces significant plasma exudation alone. The first substance increases the permeability of blood vessel walls, and the second produces vasodilatation and potentiation of plasma exudation. (Evidence that vascular permeability is increased before the generation of vasodilator mediator has been presented previously [11].) The endogenous vasodilator substance appears to be a PG; indomethacin inhibits its synthesis, removes its potentiating activity, and thus suppresses plasma exudation. Indomethacin, however, does not suppress the generation of endogenous permeability-increasing mediator, so that PGE_1 is still able to potentiate plasma exudation even in the presence of indomethacin.

Contribution of Histamine and Bradykinin to Zymosan-induced Edema. The results de-

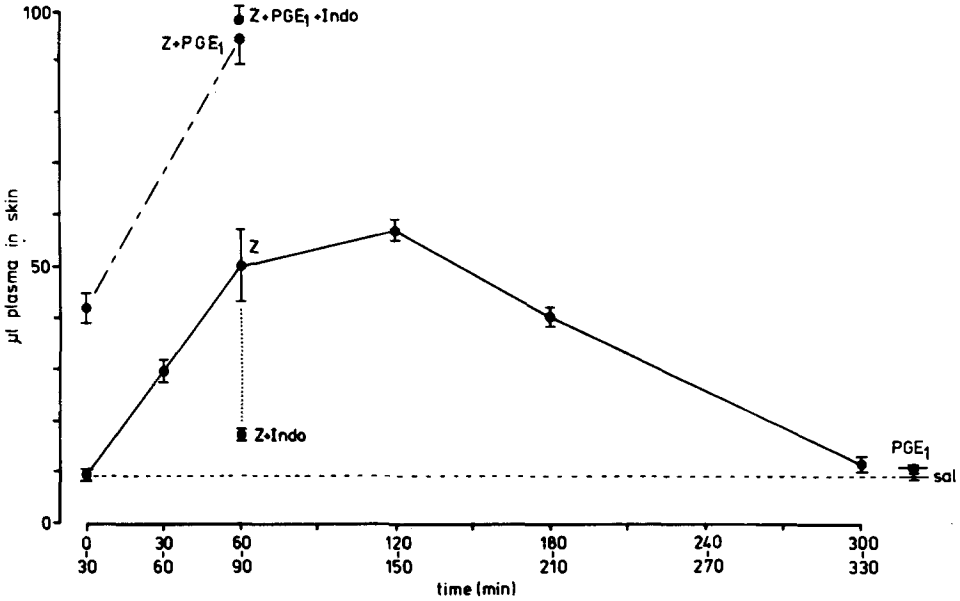

FIG. 1. Time-course of zymosan-induced plasma exudation. Zymosan (Z; 100 µg/0.1 ml) was injected intradermally at various intervals before an intravenous injection of ^{131}I-albumin. Drugs or saline were then injected locally in 0.1-ml vol, and exudation was measured over periods of 30 min as shown on the abscissa. Exudation reached a peak 1–3 h after injection of zymosan. Local injections of indomethacin (Indo; 1 µg added to the zymosan and a further 1 µg immediately after administration of isotope) suppressed the plasma exudation induced by zymosan. Local injection of PGE_1 (100 ng/0.1 ml administered immediately after isotope injection) potentiated the exudation response to zymosan. Each point represents the mean ± SEM of six replicate injections. The dashed line represents the response to two injections of saline with a 60-min interval; the small increase produced by addition of PGE_1 to the second injection is shown.

scribed above indicate that a substance is generated in the skin which acts synergistically with endogenous, or exogenous, vasodilator PG to produce inflammatory edema. Because both histamine and bradykinin are potent vascular permeability-increasing substances whose effects can be similarly enhanced by PG (14, 15), the possibility that these substances are generated in the skin was investigated. Fig. 2 shows the effects of a locally injected antihistamine, mepyramine maleate (an antagonist of H_1 receptors), on zymosan-induced plasma exudation in rabbit skin. From the results, it is clear that the antihistamine produced only a limited suppression of plasma exudation. Mepyramine was equally ineffective in suppressing potentiated responses produced by zymosan and PGE_1. On the contrary, responses to intradermally injected histamine and responses to histamine mixed with PGE_1 were effectively suppressed by mepyramine. These results indicate that the receptors involved in the enhanced vascular permeability responses to histamine in rabbit skin are H_1 receptors, but that histamine is unlikely to be a major permeability-increasing mediator generated in response to zymosan.

Similar experiments were carried out using aprotonin (Trasylol), which inhibits kinin formation by tissue and plasma kallikreins. This drug produced no significant inhibition of the responses to zymosan, or zymosan and PGE_1, but suppressed

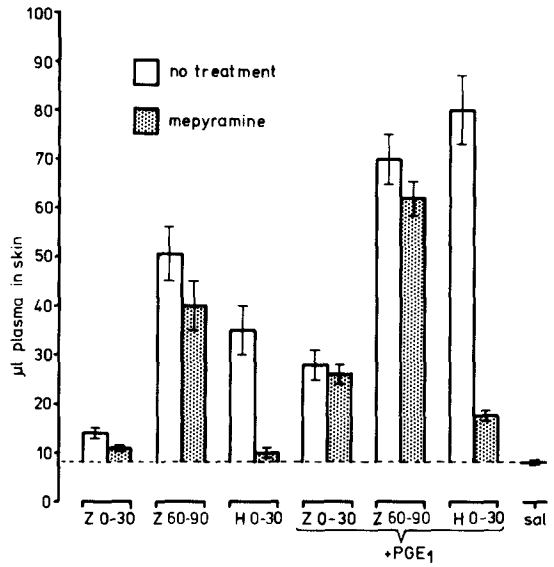

FIG. 2. The effect of mepyramine on zymosan-induced plasma exudation. Zymosan (Z; 100 µg/0.1 ml) was injected 60 min before (60- to 90-min response) or immediately after (0- to 30-min response) an intravenous injection of ^{131}I-albumin, and exudation was measured over a period of 30 min. Sites treated with mepyramine are shown as shaded columns. Mepyramine maleate (1 µg base/0.1 ml) was injected mixed with the zymosan; in the case of the 60- to 90-min response, a further intradermal injection was given immediately after the intravenous injection of isotope. The effect of mepyramine on the 0- to 30-min response to histamine (H; 2.5 µg/0.1 ml) is shown for comparison. All treatments were tested with and without PGE_1 (100 ng/0.1 ml injected immediately after isotope administration). Responses to zymosan, with and without PGE_1, were resistant to mepyramine treatment. In this experiment, the only response to zymosan that was significantly (unpaired Student's t test) affected by mepyramine was that for 0–30 min without PGE_1. In two of a further six experiments, mepyramine produced a small significant reduction of the later part of the response. In contrast, mepyramine virtually abolished the responses to histamine, with and without PGE_1, in all experiments. Each column represents the mean ± SEM of six replicate injections. The dashed line represents the response to two injections of saline (Sal) with a 60-min interval.

responses to a standard preparation of tissue kallikrein (Fig. 3). These results suggest that kinins are not involved in the response to zymosan.

Generation of a Vascular Permeability-increasing Factor in Blood Plasma during Incubation with Zymosan. The possibility that the unknown endogenous permeability-increasing mediator was a product of blood plasma was investigated by testing rabbit plasma after incubation with zymosan. Fresh plasma was incubated for 30 min with zymosan at concentrations up to 10 mg/ml, and the zymosan was removed by centrifugation. Samples of plasma were then injected into rabbit skin and responses, in terms of plasma exudation, measured over a period of 30 min. As shown in Fig. 4, no significant plasma exudation was produced by these plasma samples. When PGE_1 was mixed with these samples, plasma which had been incubated with zymosan produced marked dose-related plasma exudation whereas plasma incubated without zymosan produced little plasma exudation. The activity generated in the plasma (designated PAPA or plasma activation permeability agent [8, 9]) was similar to the endogenous permeability-increasing mediator generated in skin in response to injected zymosan in that PAPA produced significant plasma exudation only when in the presence of a vasodilator PG. It is clear that, whereas injection of zymosan into skin results in the

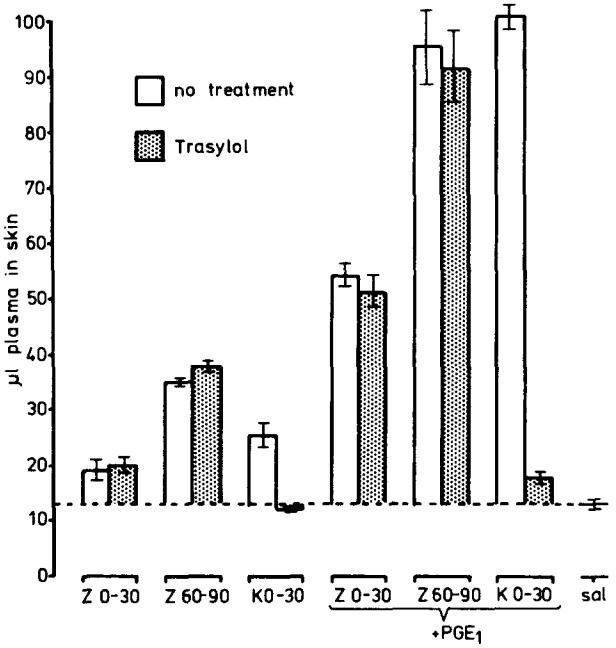

FIG. 3. The effect of Trasylol on zymosan-induced plasma exudation. Zymosan (Z; 100 µg/0.1 ml) was injected 60 min before (60- to 90-min response) or immediately after (0- to 30-min response) an intravenous injection of ^{131}I-albumin, and exudation was measured over a period of 30 min. Sites treated with Trasylol are shown as shaded columns. Trasylol (1 µg/0.1 ml) was injected mixed with the zymosan; in the case of the 60- to 90-min response, a further intradermal injection was given immediately after the intravenous injection of isotope. The effect of Trasylol on the 0- to 30-min response to kallikrein (K; 150 ng/0.1 ml) is shown for comparison. All treatments were tested with and without PGE_1 (100 ng/0.1 ml injected locally immediately after administration of isotope). Trasylol had no significant effect on zymosan-induced plasma exudation in the periods 0–30 and 60–90 min with, and without, PGE_1. Trasylol, however, virtually abolished the responses to kallikrein with and without PGE_1. Each column represents the mean ± SEM of six replicate injections. The dashed line represents the response to two injections of saline (Sal) with a 60-min interval.

generation of permeability-increasing activity and vasodilator activity, intradermal injection of zymosan-activated plasma produces only the permeability-increasing activity. For this reason, in all subsequent experiments, samples derived from zymosan-activated plasma were mixed with the vasodilator PGE_1 before testing in the skin.

The generation of permeability-increasing activity was inhibited by either heating the plasma at 56°C for 30 min or addition of EDTA (10 mM, pH 7.4) before incubation with zymosan. The same heat treatment or addition of EDTA after removal of zymosan had no effect on activity. Addition of EGTA (10 mM, pH 7.4), Trasylol (100 µg/ml), or SBTI (100 µg/ml) before incubation with zymosan did not inhibit the generation of permeability-increasing activity. The response to activated plasma (and to histamine and bradykinin) varied between individual rabbits, but permeability-increasing activity was observed in each of 54 different plasma samples activated with zymosan (1 mg/ml) tested in the same number of rabbits. No difference between homologous and autologous plasma samples was observed.

Demonstration that PAPA Has an Action Independent of Histamine Release. The results described above suggest that PAPA could be an anaphylatoxin generated by activation of the complement system. Most previous work suggests that anaphylatoxin

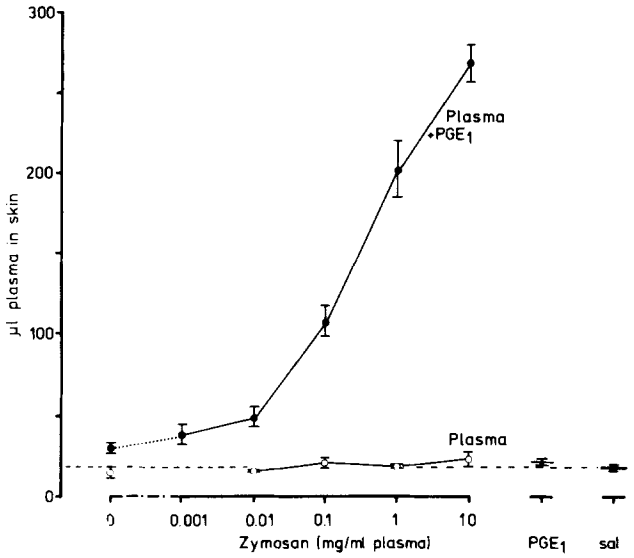

FIG. 4. The generation of permeability-increasing activity by incubation of blood plasma with zymosan. Heparinized rabbit plasma was incubated for 30 min at 37°C with various doses of zymosan, centrifuged to remove the zymosan, and injected intradermally with, or without, PGE_1 (100 ng/0.1 ml). Plasma exudation was measured by the accumulation of previously administered ^{131}I-albumin over a period of 30 min. Plasma incubated with zymosan produced a marked dose-related plasma exudation when tested in the presence of PGE_1; plasma incubated without zymosan produced only a small response in the presence of PGE_1. No exudation was produced by any of the plasma samples tested in the absence of PGE_1. Each point represents the mean ± SEM of six replicate injections. The dashed line represents the response to an injection of saline (Sal); the small increase produced by addition of PGE_1 to saline is shown.

increases vascular permeability by release of histamine (4–6); although it has been reported that responses to hog C5a are only partially dependent on histamine when tested in guinea pig skin (16). Fig. 5 shows the results of an experiment designed to investigate if PAPA increases vascular permeability by a mechanism dependent on histamine release. All responses were measured in the presence of PGE_1. Plasma activated with zymosan (1 mg/ml, as in all subsequent experiments) was mixed with a range of doses of the antihistamine, mepyramine maleate, and tested in the skin. Histamine (also with PGE_1) was mixed with the same doses of mepyramine and tested in the same experiment. It is clear that mepyramine at doses of 1 and 3 µg virtually abolished the response to histamine but had only a limited suppressive effect on a matched response to activated plasma. In a series of experiments, the suppression of responses by mepyramine (1 µg/0.1 ml) was 26 ± 3%, $n = 21$ samples of activated plasma (mixed with PGE_1) tested in the same number of rabbits.

The results obtained with mepyramine fulfill a further criterion necessary to suggest that PAPA is the permeability-increasing mediator generated in skin in response to intradermally injected zymosan.

Characterization of PAPA. Gel filtration of zymosan-activated plasma on a column of Sephadex G-100 revealed a single peak of permeability-increasing activity (assayed in the presence of PGE_1) corresponding with a molecular weight of 14,000–22,000 (Fig. 6). No permeability-increasing activity was detected when plasma incubated in the absence of zymosan was fractionated by this procedure.

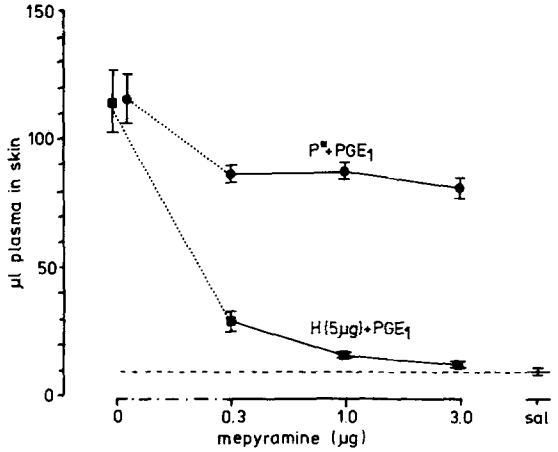

FIG. 5. A comparison of the effect of mepyramine on plasma exudation produced by zymosan-activated plasma and by histamine, both in the presence of PGE_1. Rabbit plasma was activated by incubation with zymosan (1 mg/ml) for 30 min at 37°C and centrifuged to remove the zymosan. Activated plasma (P*), or histamine (H; 5 µg/0.1 ml), was mixed with PGE_1 (100 ng/0.1 ml) and various doses of mepyramine, and then injected intradermally. Plasma exudation was measured by the accumulation of previously administered ^{131}I-albumin over a period of 30 min. Activated plasma and histamine produced responses of similar size in the absence of mepyramine. However, mepyramine almost totally abolished the response to histamine but had only a partial effect on the response to activated plasma. Each point represents the mean ± SEM of six replicate injections. The dashed line represents the response to an injection of saline.

The permeability-increasing activity was completely retained by the cation exchanger CM-Sephadex C-25 at pH values of 6.0 and below, and was eluted with 0.5 M ammonium formate. Gel filtration of the lyophilized ammonium formate fraction on a column of Sephadex G-75 superfine revealed two small peaks of material (detected by absorbance at 217 nm) corresponding with proteins of molecular weights 11,000 and 18,000. Permeability-increasing activity was eluted in fractions corresponding with a molecular weight of 18,000 (Fig. 7). This value is similar to those previously determined by gel filtration for the anaphylatoxin derived from the fifth component of complement, i.e., human C5a, 17,500 mol wt (2), and rabbit C5a, 15,000 mol wt (1).

To obtain material for further characterization, PAPA was adsorbed from 400- to 800-ml vol of zymosan-activated plasma onto CM-Sephadex C-25 (100 ml plasma/column of 1.5 × 2 cm), eluted with ammonium formate, pooled, lyophilized, chromatographed on Sephadex G-100, and concentrated by a second lyophilization (Materials and Methods). Material prepared in this way was used in all subsequent experiments.

PAPA, and a control sample prepared from plasma which had been incubated without zymosan, was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in a 5–20% acrylamide gradient gel using a discontinuous Tris:HCl buffer system (17). The major protein band, which was absent in the control sample, corresponded with a molecular weight of 13,000 with a mobility between that of cytochrome *c* (12,400 mol wt) and ribonuclease (13,700 mol wt). The difference in molecular weight, as determined by two different physical methods, is consistent with that reported for human C5a (3). SDS-gel electrophoresis also revealed a second, but

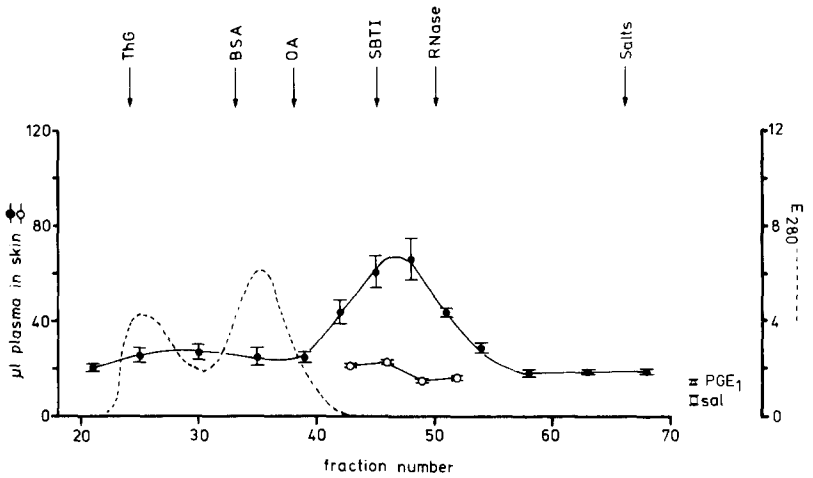

FIG. 6. Gel filtration of zymosan-activated plasma on Sephadex G-100. Rabbit plasma was activated by incubation with zymosan (1 mg/ml) for 30 min at 37°C, centrifuged to remove the zymosan, and a 7-ml sample chromatographed on a column (2.6 × 65 cm) of Sephadex G-100 in 0.9% saline. Fractions collected were each of 5.2 ml in volume. Absorbance (extinction units [E]) at 280 nm (---) shows two peaks. Fractions were mixed with PGE_1 (100 ng/0.1 ml) and injected intradermally. Plasma exudation, measured by the accumulation of previously administered ^{131}I-albumin over a period of 30 min, revealed a single peak of permeability-increasing activity (●) corresponding with a molecular weight of 14,000–22,000. Fractions obtained from plasma incubated without zymosan (○) showed no permeability-increasing activity. Each point represents the mean ± SEM of replicate injections ($n = 6$ for fractions 42–54 from activated plasma; $n = 3$ for all other sites). The responses to injections of saline (Sal) (□) and PGE_1 (△) are shown. The arrows indicate the positions of calibration proteins and salts (Materials and Methods).

considerably less intense, protein band (11,000 mol wt); this component was also present in the control sample. There was no detectable loss of permeability-increasing activity when PAPA was incubated in fresh rabbit plasma (1 h at 37°C), in phosphate-buffered saline pH 7.2 (4 h at 37°C or 1 h at 56°C) or in dilute hydrochloric acid (pH 3, 1 h at 37°C). Incubation at 100°C, pH 7.2, resulted in a 30% loss of activity after 10 min and a total loss of activity after 1 h. Total loss of activity was also obtained after incubation of PAPA with 2-mercaptoethanol (0.1 M, 4 h at 37°C). These characteristics of PAPA are similar to those of C5a of other species (3).

The following additional studies show that purified PAPA is highly active on polymorphonuclear (PMN) neutrophils: (*a*) PAPA produces a dense infiltration of PMN neutrophils when injected into rabbit skin (T. J. Williams and P. J. Jose. Unpublished data.); (*b*) PAPA induces chemokinesis with mean effective dose (ED_{50}) of 1.2×10^{-9} M and aggregation of human peripheral blood PMN neutrophils (M. A. Bray and A. W. Ford-Hutchinson. Unpublished data.); and (*c*) PAPA induces lysosomal enzyme release from rabbit PMN neutrophils (ED_{50}, 1.4×10^{-8} M) (J. P. Bennett. Unpublished data.). Similar activities on leukocytes have been observed previously with C5a in the rabbit (1, 18) and other species (19, 20).

Comparison of PAPA with Highly Purified Hog C5a. Activated rabbit plasma has little spasmogenic activity on the guinea pig ileum. However, the results described above suggest that the permeability-increasing activity generated in rabbit plasma is C5a, which corresponds with the classical anaphylatoxin of other species. The experiment shown in Fig. 8 was designed to verify further the identification of PAPA as C5a.

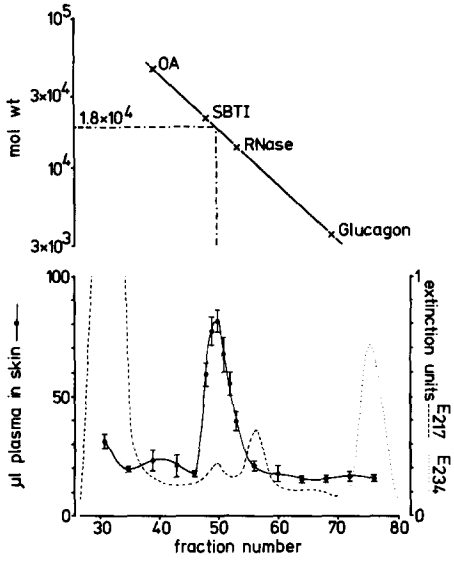

FIG. 7. Determination of the molecular weight of PAPA. Rabbit plasma (34 ml) was activated by incubation with zymosan (1 mg/ml) for 30 min at 37°C, centrifuged, and concentrated by adsorption on a column (1.5 × 2 cm) of CM-Sephadex C-25 at pH 5.5–6.0. The column was washed with buffer, and then PAPA was eluted with ammonium formate, lyophilized, dissolved in 1.5 ml saline, and chromatographed on a column (1.5 × 87 cm) of Sephadex G-75 superfine in 0.9% saline. Fractions collected were each of 2.0 ml in volume. Absorbance (extinction units [E]) at 217 nm (dashed line) shows a large peak in the region of the void volume and two smaller peaks at fractions 50 and 56. The salt peak was located by absorbance at 234 nm (dotted line). Fractions were diluted threefold with saline, mixed with PGE_1 (100 ng/0.1 ml), and injected intradermally. Plasma exudation (●), measured by the accumulation of previously administered ^{131}I-albumin over a period of 30 min, showed a peak of activity corresponding with the small absorbance peak at fraction 50. Each point represents the mean ± SEM of replicate injections ($n = 6$ for fractions 48–56; $n = 3$ for all other sites). The response to saline was 10.8 ± 0.4 µl and that to PGE_1 14.0 ± 0.9 µl. The upper part of the figure shows a plot of molecular weight vs. fraction number for some calibration proteins (Materials and Methods). The molecular weight of PAPA is estimated by gel filtration to be 18,000.

PAPA was compared with a standard preparation of hog C5a (supplied by Professor W. Vogt) that was purified on the basis of its spasmogenic activity (21). Both materials showed similar activity when injected into rabbit skin, i.e., both produced little plasma exudation in the absence of PGE_1; both produced dose-related plasma exudation when in the presence of PGE_1, and both produced responses that were similarly resistant to the antihistamine, mepyramine maleate.

These results support the identification of PAPA as C5a. However, the carboxyl terminal arginine of C5a is rapidly removed by plasma carboxypeptidase, leaving the spasmogenically inactive human C5a des Arg (22–24) but spasmogenically active hog C5a des Arg (25). PAPA was generated in rabbit plasma in the absence of an inhibitor of carboxypeptidase, as was the hog C5a used in the experiment described above. PAPA is, therefore, most likely to be C5a des Arg.

Comparison of the Duration of Action of PAPA and Bradykinin in Skin. PAPA is stable in plasma but, in contrast, the permeability-increasing activity of bradykinin (a polypeptide inactivated by carboxypeptidase) is abolished by a short incubation in fresh rabbit plasma (10 min at 37°C). Because of these findings, we investigated the possibility that the duration of action of PAPA in the skin is different from that of

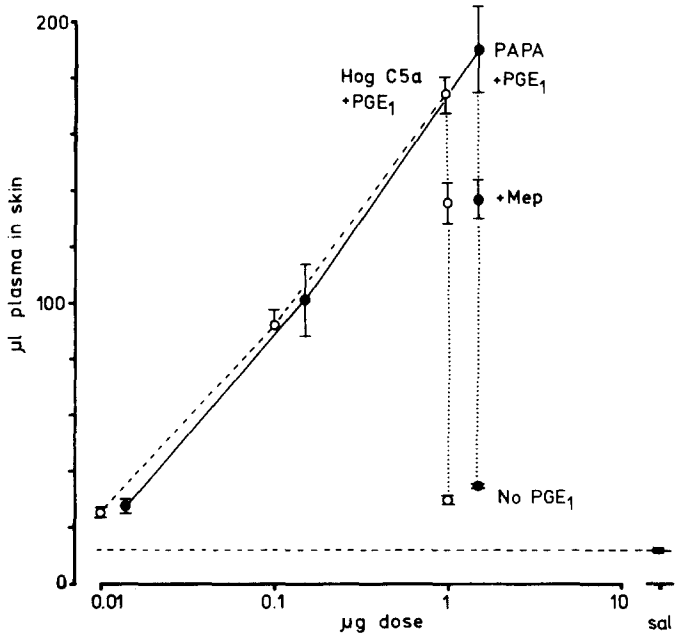

FIG. 8. Comparison of rabbit PAPA with pure hog C5a. PAPA (●) was prepared as described in the text and hog C5a (○) was supplied by Professor W. Vogt. Plasma exudation was measured by accumulation of intravenously injected ^{131}I-albumin over a period of 30 min after intradermal injections. The responses to both materials were very similar; both produced little exudation in the absence of PGE_1; both produced dose-related exudation in the presence of PGE_1 (100 ng/0.1 ml), which were similarly resistant to mepyramine (Mep; 1 µg/0.1 ml). Each point represents the mean ± SEM of six replicate injections. The horizontal dashed line represents the response to an injection of saline. In this experiment, mepyramine reduced the response to histamine (2.5 µg/0.1 ml) plus PGE_1 by 85%.

bradykinin. Fig. 9 shows the results of such an experiment. Bradykinin and PAPA were injected intradermally at intervals before the intravenous injection of labeled albumin. PGE_1 was then injected locally, and exudation was measured over a period of 30 min. It was apparent that bradykinin was rapidly inactivated ($t_{1/2}$ = 4.5 min, in this experiment; 5.9 ± 0.7 min, n = seven experiments) whereas PAPA still produced marked responses more than 2 h after its injection ($t_{1/2}$ = 101 min, in this experiment; 89 ± 11 min, n = eight experiments). Histamine produced acute responses similar to those elicited by bradykinin.

These experiments further distinguish the activity of PAPA from that of bradykinin and histamine.

Comparison of the Potencies of PAPA, Bradykinin, and Histamine. Fig. 10 shows a comparison of the potencies of PAPA, bradykinin, and histamine in inducing plasma exudation over a 30-min period in rabbit skin. In the presence of PGE_1, PAPA, at the stage of purification described here, was 40 times as active as bradykinin and 160 times as active as histamine. The range of potencies found in six different rabbits using three different PAPA preparations was PAPA 5–110 times more potent than bradykinin and 60–2,000 times more potent than histamine. The wide ranges in potency ratios are partly a consequence of differential sensitivity of the skin microvasculature of individual rabbits to the different agents. In addition, it was noticeable

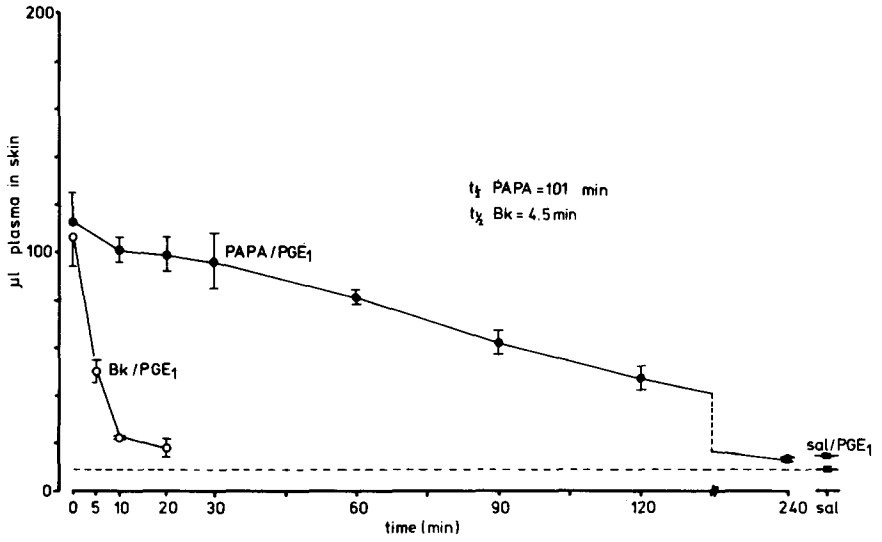

FIG. 9. A comparison of the duration of action of PAPA and bradykinin (BK) in rabbit skin. PAPA (520 ng protein/0.1 ml) and bradykinin (250 ng/0.1 ml) were injected intradermally at various intervals(shown on the abscissa) before an intravenous injection of ^{131}I-albumin. PGE_1 (100 ng/0.1 ml) was then injected locally, and plasma exudation was measured over a period of 30 min. This experimental design avoids the complication of the effect of PGE_1 inactivation in the skin. The response to bradykinin (○) was evanescent, the time interval for loss of half the response ($t_{1/2}$) being 4.5 min. In contrast, the response to PAPA (●) was still apparent when PGE_1 was administered 2 h after injection of PAPA ($t_{1/2}$ = 101 min). Each point represents the mean ± SEM of six replicate injections. The dashed line represents the response to two injections of saline with a 60-min interval; the small increase produced by addition of PGE_1 to the second injection is shown.

that the slope of the dose-response curve to bradykinin was often more shallow than that to PAPA.

When the three agents were tested in the absence of PG (at doses which produced comparable responses in the presence of PGE_1), bradykinin and histamine evoked plasma exudation but the response to PAPA was very small. This is considered to be due to the intrinsic vasodilator activity of bradykinin and histamine in rabbit skin (15). In contrast, PAPA was found to produce no significant change in blood flow, as measured by the clearance of intradermally injected ^{133}Xe. (The results of this experiment are given in the legend to Fig. 10.) Thus, a vasodilator must be added to PAPA in order to elicit significant plasma exudation. PGE_2, PGI_2, arachidonic acid, and adenosine, each of which produces an increase in blood flow in rabbit skin (11, 15) similar to that produced by PGE_1, also potentiated the plasma exudation response to PAPA. These results emphasize that, if PAPA is generated in the skin in response to injected zymosan, a vasodilator substance (probably a PG) must be generated concomitantly in order to produce the observed plasma exudation.

Discussion

Anaphylatoxin was originally proposed to be the mediator of systemic anaphylaxis (7). It was postulated that the bacteriolytic factor present in normal serum, complement, cleaved anaphylatoxin from the antigen, a process facilitated by the combination of antigen with antibody. Later, it was realized that anaphylatoxin was a product

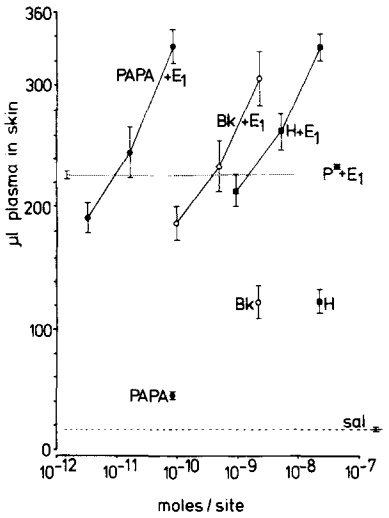

FIG. 10. Comparison of the potency of PAPA, bradykinin, and histamine in inducing plasma exudation in rabbit skin. PAPA (●), bradykinin (Bk) (○), and histamine (H) (■) were injected intradermally, and exudation was measured by the accumulation of previously administered ^{131}I-albumin over a period of 30 min. When compared in the presence of PGE_1 (E_1; 100 ng/0.1 ml), PAPA (at the stage of purification described and using a molecular weight of 18,000) was 40 times more active than bradykinin and 160 times more active than histamine. In the absence of PGE_1, bradykinin and histamine did induce plasma exudation but the response to PAPA was very small. Each point represents the mean ± SEM of six replicate injections. The dashed line represents the response to an injection of saline (Sal). The dotted line represents the response to an injection of activated plasma (P*) (1 mg zymosan/ml) plus PGE_1; potency ratios were determined at this level. Bradykinin and histamine both have some intrinsic vasodilator activity (15) that could account for their ability to induce plasma exudation alone. However, PAPA, which produced little plasma exudation when tested alone, had no significant effect on blood flow as assessed using a ^{133}Xe clearance technique (10, 11). The results in terms of percent increase (+) or decrease (−) in blood flow for six replicate sites were: PAPA (1.2×10^{-12} mol), −12.0 ± 5.0%; PAPA (1.2×10^{-11} mol), −9.1 ± 7.9%; and PAPA (1.2×10^{-10} mol), −18.6 ± 6.2%. None of these results was significantly (unpaired Student's t test) different from saline injected control sites (0 ± 6.2%). In the same experiment, norepinephrine (6×10^{-11} mol) produced a marked reduction in blood flow: −77.2 ± 1.7%; and PGE_2 (3×10^{-10} mol) produced a marked increase in blood flow: +164.3 ± 14.9%.

derived from complement itself and could be generated from serum (26). After the discovery that systemic anaphylaxis could be accounted for by a sensitization of cells (27, 28), theories involving anaphylatoxin were largely discarded, and the conceptual difficulties involved in the evolution of an endogenous toxin were not pursued.

With the later discovery that anaphylatoxin was a histamine releaser (29), it was possible to link essential features of inflammation, vasodilatation and edema, with the complement system. This link was considerably strengthened by the discovery that anaphylatoxins, now known to be cleavage products of C3 and C5, had powerful chemotactic activity for leukocytes in vitro (1, 19, 20). Histamine release is still widely held to be the link between complement activation and the changes in blood vessels that occur in inflammatory reactions. However, this proposed mechanism severely restricts the general importance of complement to vascular responses because antihistamines have very limited effects in inflammatory reactions seen clinically or induced experimentally in animals. The results presented here demonstrate that complement activation can lead to increased vascular permeability by a mechanism independent

of histamine release and suggest that the important substance in this respect is C5a des Arg.

We have attempted to analyze the events occurring in tissues after the introduction of inflammatory material. This study was restricted to a nonallergic model, but we consider that the results obtained here have wider implications. The conclusions of this study are summarized below.

Intradermal injection of zymosan leads to plasma exudation, which is resistant to antihistamines and to inhibitors of kinin formation. Plasma exudation results from the formation of two chemical mediators, both of which are necessary for plasma exudation to proceed (11, 15). The first mediator increases vascular permeability, and the second is a vasodilator substance that greatly potentiates the plasma exudation induced by the first mediator. The vasodilator substance is probably a PG because its generation is suppressed by inhibitors of PG synthesis, and its action is mimicked by certain prostaglandins. (Previous experiments [11] suggest that the enzymes necessary for PG synthesis are normally active in the skin and that blood flow can be regulated in an inflammatory reaction by controlling the liberation of the substrate, arachidonic acid.) The evidence presented in this paper suggests that the mediator that is involved in increasing vascular permeability is a product of complement activation in extravascular fluid. Incubation of blood plasma with zymosan results in the generation of permeability-increasing activity. However, no vasodilator PG (or stable arachidonic acid-releasing substance) is generated by this procedure. For this reason, a PG has to be added to the activated plasma in order to produce plasma exudation, thus simulating the conditions pertaining when zymosan is injected directly into skin.

The permeability-increasing activity generated in plasma (PAPA) was purified and found to correspond with the major chemotactic activity in complement-activated rabbit plasma, C5a (1), i.e., it produced a dense infiltration of PMN neutrophils when injected into rabbit skin and was very active in three tests on PMN neutrophils in vitro. The physicochemical characteristics of PAPA were shown to be similar to the C5a anaphylatoxin of other species. However, PAPA was found to have only weak spasmogenic (classical anaphylatoxic) activity on the guinea pig ileum and was, therefore, compared with a standard preparation of hog C5a that was purified on the basis of its spasmogenic activity (21). The two materials produced similar responses, in terms of plasma exudation in rabbit skin: both were dependent on the presence of a vasodilator PG, and both showed a high resistance to the antihistamine, mepyramine.

PAPA was stable in plasma and, therefore, by analogy with other species, is presumed to be C5a devoid of its carboxyl terminal arginine, i.e., C5a des Arg. The stability of PAPA in plasma, and presumably in tissue fluid, may contribute to the high potency of the material when compared (in the presence of a vasodilator PG) with that of the established permeability-increasing compounds, bradykinin and histamine. It was observed that the activity of PAPA was of remarkably long duration in the skin when compared with the evanescent responses produced by bradykinin and histamine.

From these results we can postulate the initial series of events occurring when microorganisms enter tissues of the nonsensitized animal. The first event is recognition of foreign cell walls, a process which involves complement present in extravascular tissue fluid. Complement-mediated lysis of the foreign cells can then proceed very

quickly, the degree of cell killing depending on the defense mechanisms of the microorganism itself. Because the quantity of extravascular tissue fluid (and therefore the quantity of complement) is limited, a supply of plasma to the tissue in proportion to the stimulus size (i.e., proportional to the number of microorganisms) is essential. This requirement may explain the evolution of mechanisms for increasing vascular permeability utilizing one of the side products of complement activation, C5a, as a local hormone. This functional interpretation of edema is strengthened by the older observations that the maximal rate of killing of bacteria is within 2 h of injection into skin (30, 31), which corresponds with the period of maximal plasma exudation reported here.

The local extravascular generation of C5a can control vascular permeability. However, in our model, a concomitant generation of vasodilator PG is obligatory in order to produce plasma exudation. (Factors governing the release of the arachidonic acid substrate remain unknown, but none of the stable products of complement-activated plasma appear to release arachidonic acid, i.e., activated plasma does not produce vasodilation as does arachidonic acid.) Leukocytes, predominantly PMN neutrophils, accumulate over a more protracted time-course than that observed for plasma exudation. These cells are involved in the phagocytosis of material derived from the earlier cytolytic process and in killing those organisms which have resisted the cytolytic phase. Previous observations (1) suggest that C5a is the most important mediator of leukocyte accumulation derived from blood plasma. (Products of arachidonic acid derived from the lipoxygenase pathway, e.g., 5,12-dihydroxy-eicosatetraenoic acid, may also contribute to leukocyte accumulation [32].) Thus, extravascular generation of C5a may be important for both fluid and cell accumulation in the inflammatory reaction, and it is of interest that C5a des Arg appears to be highly active in both processes.

Although we have used a simple nonallergic model, we suggest that the cooperation between a PG and C5a to produce edema may be relevant to many types of inflammatory reaction. In some animal models of tissue trauma, edema is suppressed by inhibitors of PG synthesis (33), and damaged tissues have been shown to activate plasma complement in vitro (34). In acute allergic reactions, such as the Arthus reaction, edema is suppressed by inhibitors of PG synthesis (35, 36) and by depletion of complement (37). Similar interactions may occur in chronic inflammation because macrophages residing in tissues can synthesis PG (38, 39) and complement components (40, 41). Our observations have been interpreted as representing part of a defense process in which the components of the inflammatory process are assembled in order to remove a potentially hazardous stimulus, and are then disassembled after its elimination. However, the general principles expressed may be relevant to chronic, self-destructive inflammatory reactions that may reflect a failure of the chemical control systems.

Summary

Intradermal injection of zymosan into nonsensitized rabbits induces plasma exudation, which is dependent on two mediators: C5a generated in extravascular tissue fluid and a vasodilator prostaglandin generated from substrates localized in cell membranes. This relationship between the complement system and the prostaglandin synthesis system has not previously been explored, and complement activation has

generally been associated with increased vascular permeability via histamine release. We report that C5a increases vascular permeability by a mechanism that is not dependent on histamine release; however plasma exudation is virtually undetectable in the absence of a vasodilator substance. Because the permeability-increasing activity is stable in plasma, analogy with other species suggests that the activity is a result of C5a devoid of its carboxyl terminal arginine (C5a des Arg). This relates the observed permeability-increasing activity with effects on leukocytes rather than effects as an anaphylatoxin.

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