

Cutaneous basophil anaphylaxis.

Immediate vasopermeability increases and anaphylactic degranulation of basophils at delayed hypersensitivity reactions challenged with additional antigen

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Summary. Many delayed-type reactions contain large infiltrates of basophils whose function is unknown. We have studied these cutaneous basophil hypersensitivity (CBH) reactions in guinea-pigs to ascertain whether basophils that are recruited to delayed reaction sites could be triggered for immediate reactivity. We compared 24 h CBH reactions with nearby skin for immediate hypersensitivity by challenging each site with small amounts of antigen. CBH sites had augmented immediate increases in vascular permeability detected by extravasation of Evan's blue dye.

The ability to elicit this augmented anaphylactic phenomenon correlated with the local presence of basophils, and light microscopy at CBH reactions 15 min after antigen challenge showed a 50% decline in basophil counts. Electron microscopy showed that progressive anaphylactic-type degranulation of local basophils occurred within minutes following re-introduction of antigen. There was fusion of vacuoles containing granules, exocytosis of granules, and dis-

solution of granules, without ultrastructural disruption of cellular integrity.

These results establish that basophils in CBH reactions can be triggered with soluble antigen to undergo anaphylactic degranulation, with the immediate release of vasoactive mediators. We have termed this phenomenon 'cutaneous basophil anaphylaxis'. Thus, one function of basophils at sites of delayed hypersensitivity may be to provide the potential for augmented, local, immediate anaphylactic reactivity.

INTRODUCTION

It is well established that fixed tissue mast cells are of great importance in the mediation of immediate hypersensitivity reactions *in vivo* (Orange, 1973; Soter & Austen, 1976). In certain species such as humans and guinea-pigs, circulating bone marrow-derived polymorphonuclear cells called basophils resemble mast cells, but normally are not present in the tissues. Both cell types have surface Fc receptors for anaphylactic antibodies and are characterized by cytoplasmic metachromatic granules which contain vasoactive amines.

Basophils in human peripheral blood are readily accessible for detailed laboratory investigation of

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IgE mediated anaphylactic functions relevant to allergic diseases. Thus *in vitro* study of IgE mediated histamine release from basophils closely correlates with clinical allergic symptom scores, wheal and flare skin testing, and results of hyposensitization treatments (Norman & Lichtenstein, 1973; Lichtenstein, Gillespie & Bourne, 1973; Evans, Pence, Kaplan & Rocklin, 1976). However, actual *in vivo* participation of basophils in immediate hypersensitivity has only been demonstrated by Chan (1972) in systematic anaphylaxis. On the contrary, the recent discovery of large tissue infiltrates of basophils in delayed-type hypersensitivity reactions, suggests that a prominent *in vivo* role of these cells may be in reactions mediated by thymic dependent lymphocytes.

This study is concerned with the function of basophils in these cutaneous basophil hypersensitivity (CBH) reactions. We present evidence linking the participation of basophils in delayed reactions, with anaphylactic mediator release of immediate hypersensitivity. We have termed this phenomenon 'cutaneous basophil anaphylaxis' (CBA). Once basophils arrive at delayed reaction sites, local re-administration of small quantities of antigen triggers these cells for immediate release of vasoactive mediators. Fifteen minutes after further antigen challenge of CBH reactions, light microscopy showed a 50% reduction in local basophils. Electron microscopy of CBH sites fixed at 5 min intervals after antigen challenge revealed progressive anaphylactic-type degranulation of local basophils. These findings establish that functional and morphological aspects of augmented, local, immediate hypersensitivity are one result of the presence and accumulation of basophils at delayed hypersensitivity reactions.

MATERIALS AND METHODS

Animals, immunization and skin testing

Hartley strain albino guinea-pigs were obtained from the colony of the Division of Animal Care, Yale University School of Medicine. Female 250 g animals were used in all experiments. The antigen, keyhole limpet haemocyanin (KLH) (Pacific Biomarine, Venice, Calif.) and the diluent buffer (PBS) used in this study were found to be free of endotoxin. This was determined with the assistance of Dr Elisha Atkins by rectal temperature readings

following intravenous injection into rabbits. Guinea-pigs were immunized for CBH reactions by intradermal injection of 200 μ g KLH in 0.1 ml PBS into the flank skin (*primary* injection). At day six skin tests with 200 μ g KLH were injected into two widely separated sites on the opposite flank (*secondary* injections). At day 7 these secondary sites showed erythematous and slightly indurated 24 h reactions which contained numerous basophils (Askenase, Haynes, Tauben & DeBernardo, 1975).

Guinea-pigs with these 24 h CBH reactions were the starting point for all subsequent experiments. Tests for immediate hypersensitivity were performed both at 24 h CBH sites and at paired adjacent normal skin of these immune animals. Guinea-pigs were first injected intravenously via the dorsal foot vein with 1 ml of 0.5% Evan's blue dye and care was taken not to traumatically release dye into the skin. CBH sites of animals examined minutes after dye injection had a slightly increased blue colour which easily blanched with light pressure, indicating vasodilation rather than detectable extravasation of intravascular dye. Immediately following Evan's blue injection, the centre of 24 h CBH sites and adjacent normal skin were carefully injected with various reagents in PBS in a volume of 0.1 ml (*tertiary* injections). Twenty minutes later the animals were killed, flank skin was removed, and measurements were made on the inner surface of the diameter in mm of extravasated dye. Student's paired *t* test was used to compute the statistical significance ($P < 0.05$) of blueing diameters obtained at duplicate CBH skin sites compared to paired normal control duplicate skin sites.

Immunization and skin testing of controls

In control experiments, secondary skin tests consisted of two drops of 2 or 10% croton oil (Fisher Scientific Co., Pittsburgh, Pa.) in acetone: olive oil, 4:1, or intradermal injection of 100 μ g PHA-P (Difco Laboratories, Detroit, Mich.). Control tertiary skin tests were performed with 0.5 or 1.0 μ g histamine (Calbiochem Co., Berkeley, Calif.), or Compound 48/80 (1 or 4 μ g) (Burroughs Wellcome, Research Triangle, N.C.). In other experiments, animals were immunized with 100 μ g KLH emulsified with Freund's Incomplete Adjuvant (FIA) or Freund's Complete Adjuvant (FCA-H37Ra) (Difco Laboratories, Detroit, Mich.) fortified with 3 mg/ml ground *Mycobacteria tuberculosis*. Animals immunized with FIA or FCA were skin tested for

delayed reactions with 100 μg KLH at day 6. Final skin testing for immediate reactivity at normal skin and delayed reaction sites was performed 24 h later with 4 μg KLH in animals immunized with FIA, and with 8 μg KLH in animals immunized with FCA,

Histology and cell counting

Reactions were processed for quantitation of basophil infiltrates as previously described (Askenase, Haynes & Hayden, 1976b). To compare cell counts at antigen vs PBS challenged CBH sites, we placed a 2 \times 2 mm grid in the microscope ocular. The grid area covered about one third of our 180 μm diameter oil-powered (\times 1000) field. Slides were coded and twenty consecutive grid fields in the upper dermis were counted in each tissue section starting from the reaction centre and progressing towards the periphery. Islands of epidermis, hair follicles, and sweat glands were skipped. Total nucleated cells, basophils and eosinophils were counted. The Mann Whitney U test was used to compute the statistical significance ($P < 0.05$) of differences in mean cell percentages.

Electron microscopy

In preliminary experiments skin specimens were fixed by immersion in freshly prepared 0.1 M sodium cacodylate (K & K Laboratories, Plainview, N.Y.) buffered (pH 7.2) paraformaldehyde (2%) – glutaraldehyde (2.5%) (EM Sciences, Fort Washington, Pa.). However, less artefacts and also more optimal fixation of rapidly evolving *in vivo* changes were achieved by whole body perfusion with fixative. Thus, animals were killed by cervical dislocation immediately (or at intervals) after further antigen injection of CBH sites, the chest was rapidly opened and the heart was exposed. Then, the aorta was cannulated and animals were systemically perfused by gravity flow with saline, followed by perfusion with fixative diluted 1:3 with buffer. CBH reactions were then removed, diced with razor blades, and fixation was completed by immersion in full strength fixative at 0° for 5 h. Specimens were then washed five times in plain 0.1 M cacodylate buffer (pH 7.2) and subsequently post-fixed in 1.33% collidine buffered (pH 7.2 EM Sciences, Fort Washington, Pa) osmium tetroxide (Polysciences, Warrington, Pa) and stained en bloc with uranyl acetate oxalate (Fisher Scientific Co., Pittsburgh, Pa). Specimens were then dehydrated in graded alcohols and embedded in epon 812 (Ladd Research Industries, Burlington, Vt.)

One micron thick sections were cut with glass knives on an LKB Huxley Ultramicrotome, stained with azure II-methylene blue stain and inspected by light microscopy. Thin sections were cut with a diamond knife, stained with 2% aqueous uranyl acetate followed by lead citrate and examined in a Zeiss EM10B electron microscope. Multiple specimens from control (PBS injection) and experimental CBH reactions (injected with KLH and fixed at 0, 5 or 10 min) were examined from three separate experiments. To ensure that events at one skin site did not influence ultrastructural changes at another site, primary cutaneous basophil flare reactions (Askenase *et al.*, 1975) were the CBH sites chosen for electron microscopic studies, and only one immediate injection (into a CBH site vs a normal skin site) per animal was employed in these studies.

RESULTS

Augmented vascular permeability at antigen-challenged CBH Reactions

Figure 1a shows that when Evans blue dye was injected into an animal with a 24 h CBH reaction and then normal and CBH sites were injected with 0.1 ml PBS, no significant extravasation of blue dye occurred at either site. In preliminary experiments determination was made of the tertiary intradermal dose of KLH which caused in normal skin of immunized animals a minimal blueing reaction that was greater than that produced by injecting PBS into normal skin. Generally, 2 or 4 μg was chosen as the dose of KLH used to test for immediate hypersensitivity at CBH vs normal sites. Figure 1b shows that when 2 μg KLH was injected into CBH reactions there was a greatly augmented extravasation of blue dye (15.4 ± 1.6 mm) compared to normal skin (7.3 ± 0.6 mm) in twenty paired sites. Augmented increases in vascular permeability were also obtained with a tertiary challenging dose of 4 μg KLH (Fig. 1c). Animals which tended to have greater reactivity at normal skin were augmented to the greatest diameters of blueing at CBH sites.

After the injection of Evans blue dye, CBH and normal skin sites were challenged with antigen and groups of animals were killed immediately, 5, 10 and 20 min later. It was found at both sites that blueing was evident within 5 min, and was nearly complete by 10 min (Fig. 2). When Evans blue dye was injected intravenously 15 min *after* antigen

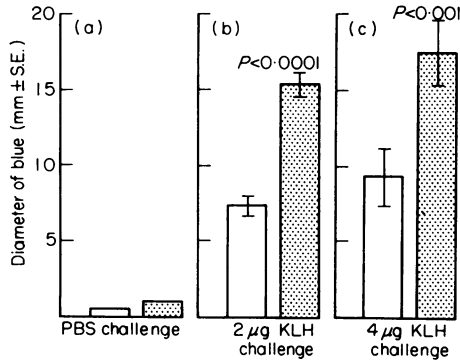


Figure 1. Augmented immediate vasopermeability at CBH reactions vs normal skin sites. CBH reactions elicited by KLH were challenged with 0.1 ml PBS alone (a) or containing 2 or 4 µg KLH (b & c). The diameter of Evans blue dye extravasated was measured 20 min later and compared to blueing at contiguous normal skin sites. Data was pooled from several experiments which involved twenty animals. Open columns, normal skin; dotted columns, 24 h CBH sites.

challenge of CBH sites, no immediate augmented local blueing was subsequently observed. Thus, augmented vascular permeability at CBH sites challenged with additional antigen was *immediate* and *transient*—and thus was anaphylactic in character.

Vascular permeability studies following antigen challenge at various basophil reactions vs non-basophil containing delayed reactions

We considered whether the phenomenon of augmented blueing by tertiary antigen injection at CBH sites was specific for reactions elicited by the

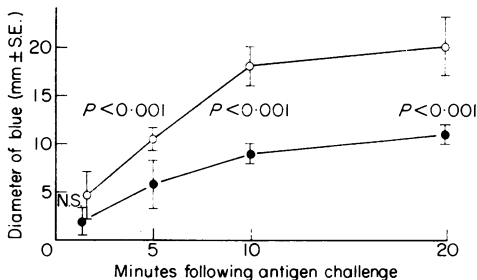


Figure 2. The time course of blueing at CBH reactions vs normal skin sites following challenge with 4 µg KLH. Each point represents the mean from four separate animals in two separate experiments. (○), 24 h CBH sites; (●), normal skin sites.

immunizing antigen. Thus, animals immunized with KLH were skin tested (secondary injections) at 6 days with 100 µg PHA, which is known to elicit basophil containing delayed responses in non-immune guinea-pigs (Stadecker & Leskowitz, 1974; Haynes & Askenase, 1977). At 24 h, Evans blue dye was given intravenously and then PHA induced reactions were injected with 2 µg KLH and compared with similar challenge of normal skin. Figure 3a shows there was a significant augmentation of vascular permeability increases at the PHA-induced CBH sites of KLH sensitized animals vs paired normal sites, and this was a highly significant result ($n=8$ pairs, $P<0.001$). When non-immune animals had similar 24 h PHA sites challenged with 2 µg and even 20 or 200 µg KLH, no similar blueing was obtained. The 24 h PHA responses of immune or non-immune animals contained 20–25% basophils. In guinea-pigs immunized with KLH no blueing was obtained at PHA or KLH induced 24 h CBH sites which were tertiary challenged by an irrelevant antigen (ovalbumin). In addition, secondary CBH sites produced by as little as 20 µg KLH in immunized animals demonstrated specific antigen induced augmented cutaneous anaphylaxis.

We tested whether a non-specific, non-basophil containing cutaneous inflammatory reaction, such as produced by croton oil and occurring in an animal immunized for CBH, would demonstrate altered vascular permeability. Figure 3b shows that a tertiary challenging dose of KLH caused the usual

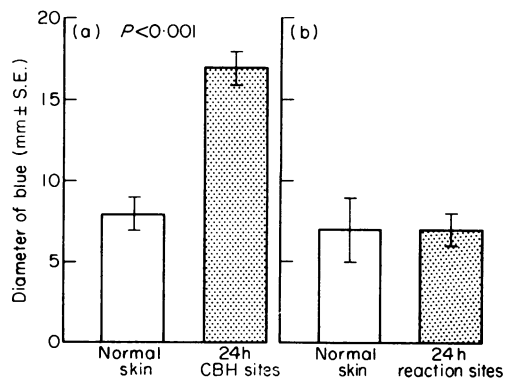


Figure 3. Tests for augmented immediate vasopermeability with 2 µg KLH at 24 h delayed reactions vs normal skin of KLH immunized guinea-pigs. Augmented local blueing was found at basophil-rich PHA responses (a), but not at basophil-poor croton oil responses (b).

blueing reactions at normal skin which were not augmented at 24 h croton oil reaction sites.

The phenomenon of augmented vascular permeability increase obtained by immediate tertiary challenges at CBH sites might be peculiar to intradermal KLH immunization. Thus, guinea-pigs were sensitized for more classical CBH by footpad injection of KLH emulsified with FIA, and as further controls another group was similarly immunized with KLH+FCA for classical delayed tuberculin-type reactions (DH). It is known that delayed reactions elicited in animals immunized with FIA and skin tested at 1 week contain many basophils, while similar KLH skin tests in animals immunized with 100 μg KLH+FCA show DH reactions with few basophils (Askenase *et al.*, 1976b). Figure 4 shows that an augmented vascular permeability increase was obtained in the CBH reactions of animals immunized with FIA, but not in the DH reactions of animals immunized with FCA. Thus, augmented local immediate hypersensitivity correlated with the presence of basophils in delayed cutaneous reactions.

Permeability studies at CBH sites challenged with vasoactive mediators

Augmented vascular permeability at CBH sites might not be due to the specific release of mediators by basophils, but due to an augmented sensitivity of the vessels to mediators released by factors operative at normal skin sites. Thus, CBH sites and normal skin sites were challenged with tertiary injection of histamine as a representative mediator, and Compound 48/80 as a non-specific releaser of

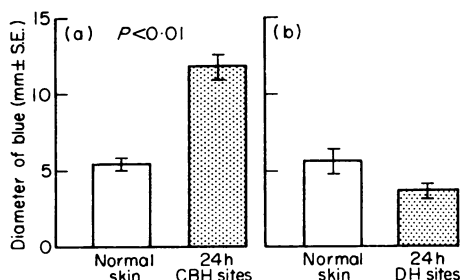


Figure 4. Tests in KLH immunized guinea-pigs for augmented immediate vasopermeability at 24 h KLH induced delayed reactions vs normal skin. Augmented local blueing was found at CBH reactions elicited in animals immunized with FIA (a) and not at basophil-poor DH reactions elicited in animals immunized with FCA (b).

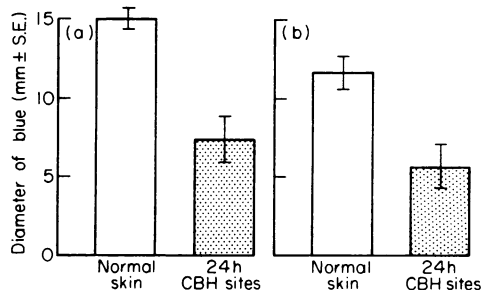


Figure 5. Tests for immediate vasopermeability caused by histamine (a) or Compound 48/80 (b) injected into normal skin vs 24 h CBH sites of immunized guinea-pigs. Hyporeactivity was noted to histamine and Compound 48/80 at CBH skin vs normal skin. (a), Histamine challenge, $P < 0.01$; (b), compound 48/80 challenge, $P < 0.01$.

mediators. The left side of Fig. 5a shows that significant blueing reactions were produced at normal skin with histamine and there were no augmented permeability increases produced by histamine at 24 h CBH sites. Figure 5b shows similar experiments performed with Compound 48/80 which also failed to elicit augmented blueing at 24 h CBH sites. In fact, there was a trend towards hyporeactivity to histamine and 48/80 at 24 h CBH sites, and this was statistically significant. It was concluded that increased sensitivity to mediators routinely released by factors resident in normal skin probably did not account for the augmented vascular permeability increases induced by antigen injection at 24 h CBH sites.

Time course of erythema and basophils at CBH reactions and of augmented vascular permeability induced by further antigen injection

Table 1 shows the time course of the diameter of erythema and accumulation of basophils at CBH reactions elicited by KLH skin testing in immunized animals. Macroscopic erythema reached a plateau at 12–24 h and declined thereafter. Basophils were minimally increased at 12 h when erythema had already reached maximal diameter. In contrast, basophil accumulations remained large at 48 h when macroscopic erythema had fallen. Developing CBH reactions were injected with an additional dose of 2 μg KLH at these various intervals and immediate vascular permeability was assessed by extravasation of Evans blue dye 20 min later. Augmented increases in vascular permeability induced by antigen injection were present at 24, 48 and even 72 h

Table 1. Time course of erythema, basophils and immediate blueing at CBH reactions

	Time (h)			
	4	12	24	48
Erythema* (mm±SE)	4±1	22±1	21±1	11±0.7
Basophils*/ 20 grid fields (% cells)	2±1 (1%)	23±5 (6%)	138±2 (25%)	220±19 (40%)
Blueing†	0%	9%	87%	42%

* Data for erythema are from 12–24 animals per interval, and basophil counts are from six animals per interval.

† At the various intervals, blueing was calculated as the % increase in mean diameter of dye extravasation (at 20 min) in CBH skin vs normal skin sites injected with an additional 2 µg KLH in six animals per interval.

(data not shown), while at 4 h and 12 h no augmented vascular permeability could be induced. Thus, when evolving CBH reactions were pulsed with further injections of a small dose of antigen, local augmented increases in vascular permeability paralleled the delayed onset and prolonged duration of basophil accumulations rather than erythema and increased local vascularity.

Light microscopic quantitative cell counts at CBH reactions receiving immediate antigen challenge

Fig. 6 shows the results of pooled data from four separate experiments in which two immunized guinea-pigs had duplicate CBH reactions induced by KLH skin testing on each flank. At 24 h one reaction per animal was challenged with 4 µg KLH and the other CBH site was injected with PBS. The animals were killed 15 min later and skin was prepared for quantitative cell counting in 20 ocular grid fields. It can be seen that a 50% decline in basophils occurred in CBH sites injected with antigen compared to controls ($P < 0.005$). In contrast, eosinophil counts rose slightly and total nucleated cells remained the same in both instances (550 ± 37 at KLH tested CBH vs 575 ± 40 at PBS tested CBH). It was concluded that immediate degranulation of basophils at sites of antigen challenge of CBH reactions could account for this rapid decline in identifiable basophils without change in total nucleated cell counts.

Electron microscopy of basophils at control CBH reactions

When CBH reactions were challenged with additional antigen, blueing was first detected by 2–5 min

and was nearly complete by 10 min (Fig. 2). Preliminary electron microscopy experiments showed that by 20 min normal intact basophils were infrequent and many basophil granules were lying between cells or were in the cytoplasm of phagocytic cells. Thus, ultrastructural alterations in basophils probably occurred extremely rapidly and were only briefly evident *in vivo*. Subsequent electron microscopic studies were performed at control CBH sites (challenged with PBS) or experimental CBH sites (challenged with 4 µg KLH) of animals which were systemically perfused with Karnovsky's fixative

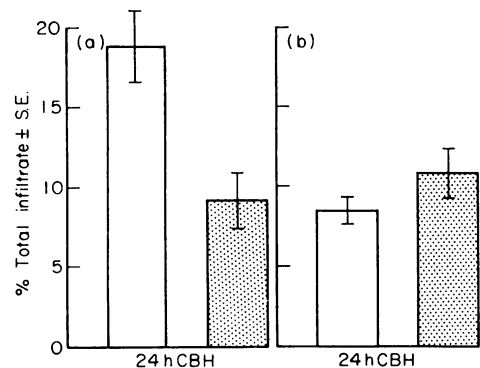


Figure 6. Quantitative counts of basophils and eosinophils in the papillary dermis of CBH reactions. In four separate experiments duplicate CBH reactions were elicited with 200 µg KLH in a total of eight guinea-pigs. At 24 h, one reaction site was challenged by further injection of 4 µg KLH. The centres of both reaction sites were harvested 20 min later and processed for cell counting. Open columns, unchallenged; dotted columns, challenged. (a) Basophils, $P < 0.005$; (b), eosinophils, $P < 0.025$.

immediately after challenge, and subsequently at 5 min intervals.

There were no differences in the basophils at control CBH reactions which were injected with PBS and examined immediately, at 5 min or 10 min later. The ultrastructure of most of these cells conformed with descriptions of normal appearing guinea-pig basophils which have been previously described in bone marrow and in some CBH reactions (Chan, 1972; Dvorak, Dvorak, Simpson, Richerson, Leskowitz & Karnovsky, 1970; Terry, Bainton & Farquhar, 1969; Dvorak, Dvorak & Karnovsky, 1972). These basophils had a rounded cytoplasmic outline, a smooth surface with occasional short surface projections, and cytoplasm containing many characteristic granules (Fig. 7a, b). These granules were composed of characteristic crystalline lattice material which completely filled the vacuole in which the granules were contained. Only minor changes were noted in the granules of control basophils. Some granules had a halo of loosely packed material at their periphery, surrounded by an enlarged perigranular space adjacent to the vacuole membrane (Fig. 7a, b). The concentric halos of perigranular space and loose granular material usually constituted less than 10% of the granule vacuole contents, but occasionally were of greater magnitude. Single or multiple outpouchings of the granule vacuole membrane were occasionally noted. These were usually empty, but sometimes were partially filled with amorphous material extending from the granule rim and protruding into this outpouching (Fig. 7a). The occurrence of several large vacuoles which contained amorphous material and were located in the region of the Golgi complex (Fig. 7b) suggests that these may be immature granules (Terry *et al.*, 1969). The cytoplasm of many basophils also contained numerous small membrane bound vesicles. These were almost always rounded, were rarely tubular in appearance, and usually appeared empty of electron dense matter (Fig. 7a, b). However, a few contained material similar to that of basophil granules, but this could not be absolutely identified as the crystal lattice material that characterizes basophil granules of guinea-pigs.

Ultrastructural observation of basophils after antigen challenge of CBH reactions

Immediately (within the first minute) after intradermal injection of KLH many basophils showed

progressive morphological phases of anaphylactic-type degranulation (exocytosis) which was accompanied by signs of surface activation. In contrast to the smooth and rounded surface of control basophils, the degranulating basophils were elongated and stretched out in several directions (Figs 9–10). Degranulating cells had a network of fine cytoplasmic surface projections which extended in many directions and resembled filopodia on activated macrophages. Another immediate change induced in basophils undergoing anaphylactic degranulation was aggregation with adjacent macrophages. This was produced by the formation of interlockings between the adjoining surfaces of activated basophils and macrophages (Fig. 10).

The earliest morphological changes in granules were noted in basophils with minimal signs of surface activation. In these cells the majority of characteristic intracytoplasmic granules were ringed with large clear halos which sometimes occupied most of the granule vacuole (Fig. 8). Communications between 'haloed' granules were numerous. An apparent subsequent phase in basophil degranulation was coalescence of granule vacuole membranes, so that two or three or more disintegrating granules were contained in a common vacuole (Fig. 9a). Many coalesced vacuoles, which contained multiple granules of decreased electron opacity, communicated with the extracellular space (exocytosis) (Figs 9b and 10). Strands of fibrillar debris clung to the surface of these dissolving granules and were also present in halos of decreasing concentration around the granules (Figs 9 and 10).

The general cell integrity of degranulating basophils was intact. The cytoplasmic membrane, and mitochondria were normal in appearance, and the nuclei were unchanged. Basophils with no evidence of degranulation were occasionally found adjacent to basophils demonstrating florid anaphylactic-type degranulation. In some degranulating basophils the majority of granules had undergone exocytosis, but a few remaining granules were still present in the cytoplasm in their individual vacuoles and had progressively larger surrounding halos (Fig. 9b). In basophils showing anaphylactic degranulation, the numerous small, round cytoplasmic membrane bound vesicles were identical to those of basophils in control CBH sites.

Five minutes after antigen challenge many basophils were similarly stretched out and had long

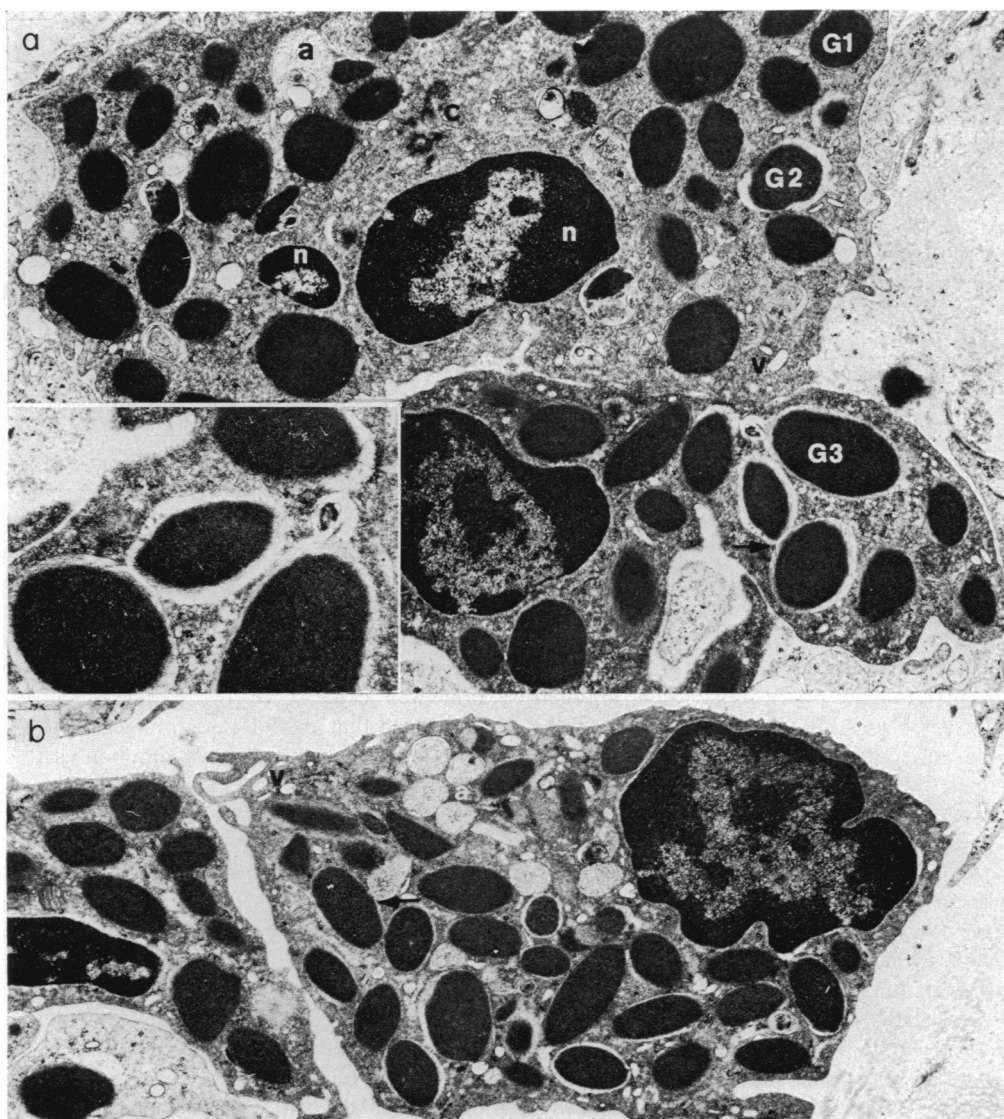


Figure 7. Control basophils in the upper dermis of a CBH reaction fixed immediately after local injection of 0.1 ml PBS. The surface of these basophils is smooth. The cytoplasm has numerous membrane bound vesicles (v) all of which are virtually devoid of electron dense material.

(a) In the upper basophil, two lobes of the nucleus (n) and a centriole (c) are visible. The majority of characteristic cytoplasmic granules completely fill their respective vacuoles. In other granules, a thin perigranular space allows visualization of the granule vacuole membrane (G1). A few granules have a larger perigranular space which constitutes 10% or less of the granule vacuole content (G2). One granule (G3) has an outpouching of the granule vacuole membrane into which fibrillar material from the granule matrix appears to be protruding (see insert). Minimal fusions of granule vacuole membranes are noted (arrow and figure insert). The basophil above also has several cytoplasmic vacuoles containing: predominantly amorphous material (a).

(b) Small halos surround many of the characteristic cytoplasmic granules. The cytoplasm of this basophil has several vacuoles containing amorphous material (a). Three of these vacuoles appear to have coalesced with other vacuoles which contain intact, or slightly haloed, characteristic cytoplasmic granules (arrow). (a) x 10000; insert x 20000 (b) x 9600.

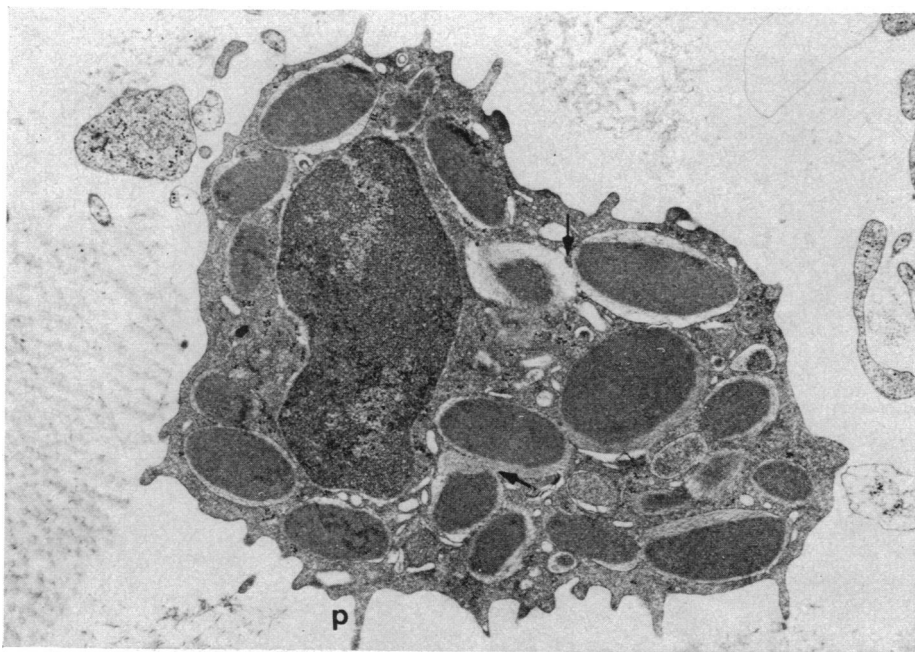


Figure 8. Early phase of basophil degranulation from experimental CBH reaction which was injected locally with 4 μ g KLH and fixed immediately thereafter. Virtually all the characteristic cytoplasmic granules are ringed with prominent halos producing enlarged perigranular spaces. Many of these halos are large and some extend for more than 50% of the granule vacuole contents. In several places, apparent fusions (arrows) between the granule vacuole membranes of these haloed granules are visible. Numerous small cytoplasmic membrane bound vesicles are empty of electron dense material. The surface of this basophil is smooth except for a few pointed projections (p). $\times 12000$.

surface filopodia. Multiple extruded granules, which were noted in progressive stages of dissociation of the characteristic crystalline bands (Fig. 11) were pooled in large vacuole spaces which communicated with the extracellular space (Figs 9 and 10). The cytoplasm of nearby macrophages contained basophil granules in various stages of degeneration, indicating that some granules released from basophils by exocytosis had undergone phagocytosis by macrophages (Fig. 12). Far fewer identifiable basophils were found at 10 min, compared to 0 or 5 min. This corroborated the quantitative basophil counts made by light microscopy (Fig. 6). Nearly all of the changes of anaphylactic degranulation occurred within a few minutes following further antigen injection, were complete by 5 min, and were much less detectable by 10 min. It was concluded that *in vivo* the ultrastructural changes of basophil degranulation by exocytosis

occurred very rapidly and were only briefly evident at CBH sites challenged by local administration of antigen.

DISCUSSION

Anaphylactic function at CBH sites. The results of this study suggest a functional role for infiltrating basophils at delayed cutaneous hypersensitivity reactions. When 24 h reaction sites containing basophils were challenged with microgram quantities of KLH in sensitized animals, immediate and transient vascular permeability occurred. This local cutaneous anaphylaxis at CBH sites was augmented over immediate changes which occurred when identical antigen doses were injected into normal skin of immune animals. Immediate hypersensitivity responses at normal skin of sensitized animals are

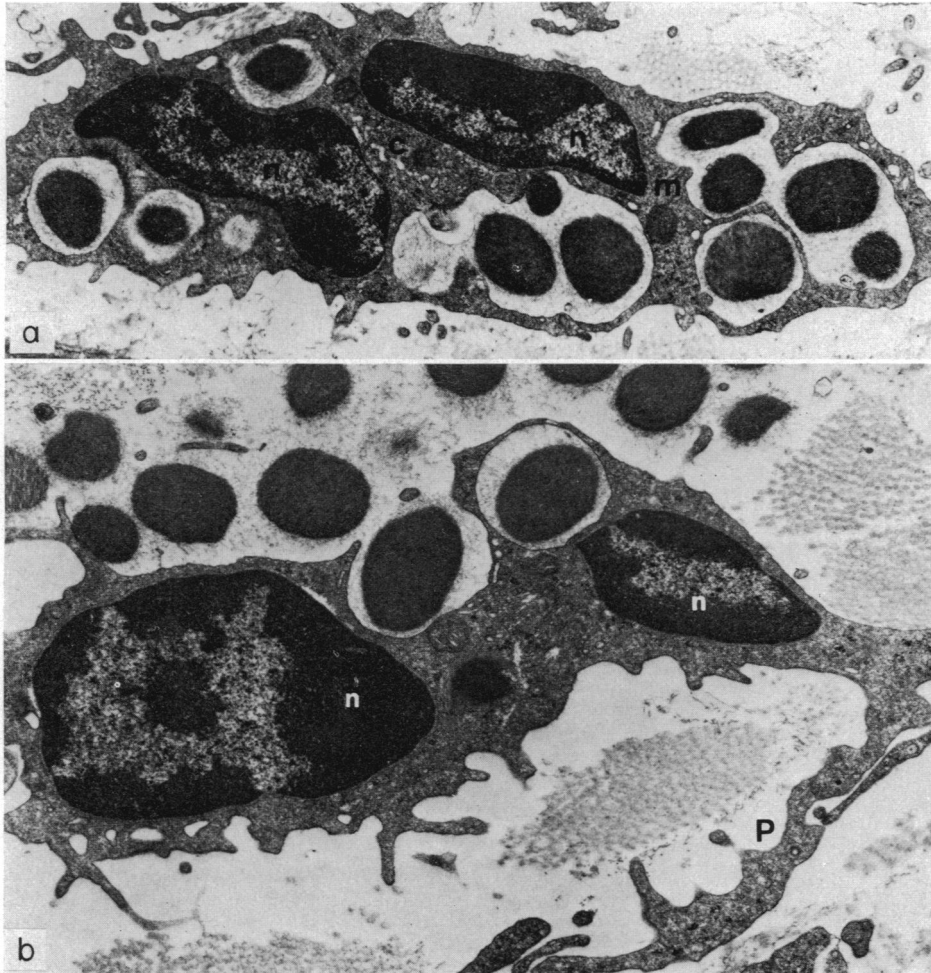


Figure 9. Further stages of degranulation in basophils from CBH reactions injected locally with KLH and fixed immediately thereafter.

(a) The cell is stretched out, rather than having the rounded or square shape of the basophils in control CBH sites (Fig. 7). A prominent centriole (c) is visible. All of the cytoplasmic granules have prominent halos. Several individual granule vacuole membranes have coalesced, placing several disintegrating granules into a common membrane bound vacuole. Some visible mitochondria (m) appear to be in good condition. The cytoplasmic and nuclear membranes are not disrupted. Two lobes of the nucleus (n) are visible. x 10700.

(b) The cell is stretched out with long complex surface filopodial projections (P) indicative of cell surface activation. At the superior border of the basophil are several granules that have apparently been extruded by exocytosis. The diffusion of crystalline material from these disintegrating granules is apparent. As the granules disintegrate, their characteristic crystalline lattice is evident. x 13900.

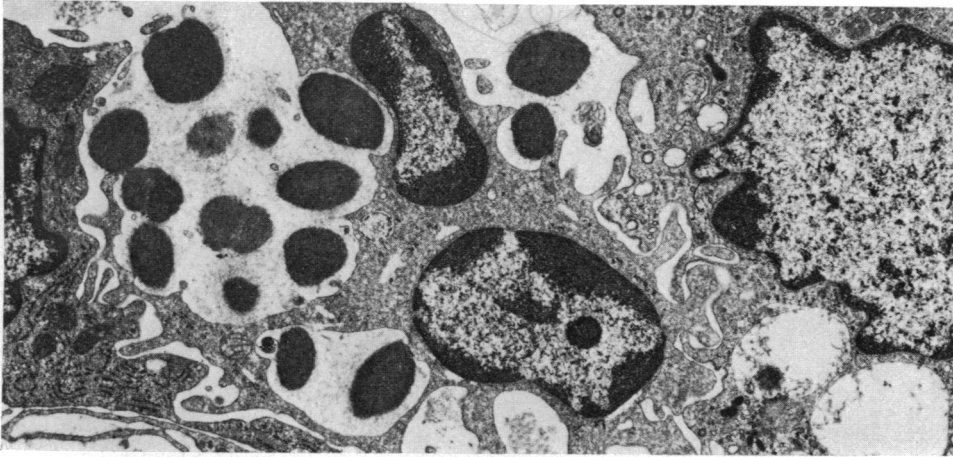


Figure 10. A degranulating basophil is seen between two mononuclear cells. The surface filopodia of the basophil interdigitate with the surfaces of the adjacent mononuclear cells, one of which (on the right) is clearly a macrophage. x 8000.

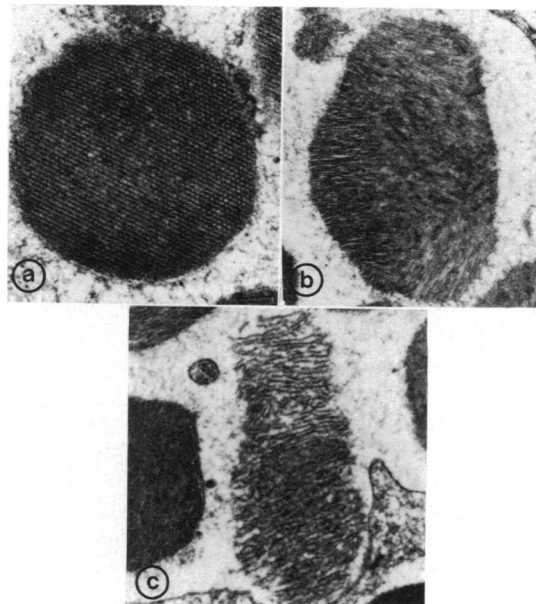


Figure 11. Higher magnification electron micrographs of basophil granules from cells in various stages of anaphylactic degranulation. (a) A well-defined and compact crystalline lattice structure can be seen in this granule. (b) This granule demonstrates loss of the uniform orientation in an irregular pattern. In the centre of the granule the plates cannot be clearly resolved and appear fuzzy. (c) There is marked separation of the individual plates with loss of the compactness of this granule. Fuzzy material can be seen surrounding the granule suggesting release of granule substance associated with the loss of organized structure. (a) x 46000; (b) x 23000; (c) x 23000.



Figure 12. A CBH reaction 5 min after reinjection of KLH. This macrophage has apparently ingested several basophil granules. While some of the ingested granules have a relatively well-preserved structure, others (arrow) show loss of integrity of the granule seen with anaphylactic degranulation. x 15800.

examples of active cutaneous anaphylaxis and are undoubtedly due to release of vasoactive mediators from mast cells following interaction of cell surface anaphylactic antibodies with antigen. Our results suggest that the anaphylactic release of vaso-active mediators at CBH sites is due to the presence of basophils and depends on active immunization. Basophil-rich reactions elicited non-specifically with PHA in non-immune animals were inactive to challenge with KLH, while sites of secondary delayed skin tests with KLH or PHA in immunized animals elicited augmented local immediate reactivity only when re-injected with KLH, the immunizing antigen. Thus, three ingredients were necessary, but no one or two were sufficient to elicit cutaneous basophil anaphylaxis (CBA): (1) active sensitization; (2) a basophil-rich delayed reaction; and (3) tertiary challenge with the immunizing antigen KLH.

Anaphylactic degranulation of basophils at CBH sites. When developing CBH reactions were pulsed at various times with further injection of antigen, the elicitation of augmented local cutaneous

anaphylaxis occurred with a delayed time course that correlated with the presence of basophils and not with erythema (Table 1). Augmented local anaphylaxis was accompanied by 50% falls in the number of local basophils identified by light microscopy 20 min following injection of antigen (Fig. 6). Study with the electron microscope revealed the pathognomic characteristics of anaphylactic-type basophil degranulation by exocytosis.

Our success at demonstrating anaphylactic changes at CBH sites challenged with KLH contrasts with negative experiments reported by Dvorak, Simpson, Bast & Leskowitz (1971) who used albumin. CBA may depend on coating of basophils with anaphylactic antibody. Thus, immunogenicity or size differences between antigens and/or the time after immunization (Dvorak, Colvin & Churchill, 1975) the sites are tested, may be determinants of the elicibility of CBA. We found that degranulation had a rapid onset and brief duration, as has previously been noted (Chan, 1972; Hastie, 1971; Hastie, Levy & Weiss, 1976). A crucial determinant of visualizing explosive and evanescent changes was systemic perfusion with fixative immediately

following tertiary antigen challenge at CBH reactions. The fact that Dvorak *et al.*, (1971) looked at antigen challenged CBH sites 1 h later could account for the lack of degranulation noted. Basophils undergoing anaphylactic degranulation showed signs of profound surface activation. Some of these changes have been previously observed *in vitro* (Hastie, 1971; Hastie *et al.*, 1976) by studying IgE mediated degranulation of human basophils exposed to pollen allergens. At CBH reactions challenged with antigen *in vivo*, multiple filopodial projections extended from the surface of activated basophils, stretched the cells out, encircled collagen bundles, and interdigitated with the surface of nearby activated macrophages. Basophil degranulation began 1–2 min after local antigen injection, first consisted of increasing halos around granules, then fusion and progressive coalescence of granule vacuoles, communication of these vacuoles with the extracellular fluid, exocytosis of granules, dissolution of the granule matrix, and preservation of general cell integrity (Fig. 8–10).

Dvorak, Dvorak & Churchill (1973) studied the interaction *in vivo* of basophils and tumour cells in immunized strain two guinea-pigs which were challenged in the peritoneal cavity with live tumour cells, and drew attention to frequent and minor changes in portions of individual basophil granules. *In situ* dissolution of individual basophil granules has also been noted to occur progressively over 24–72 h in human contact hypersensitivity reactions (Dvorak, Mihm & Dvorak, 1976a). These changes have been called 'piece-meal degranulation' (Dvorak *et al.*, 1976b; Dvorak & Dvorak, 1975). These investigators have hypothesized that portions of constituents of individual granules are slowly secreted from basophils at CBH reactions via a transport system of intracytoplasmic vesicles. They have also suggested that under some situations such a process of reversed pinocytosis could speed up and merge into exocytosis. Ultrastructural changes consistent with anaphylactic type degranulation were also noted by Dvorak *et al.* (1973) in some basophils at the CBH reactions of guinea-pigs to the tumour cells (Fig. 21), but were infrequent. This is not surprising in view of the explosive nature of *in vivo* anaphylactic basophil degranulation which has been noted in the current study.

In the CBH reactions which we have examined, minor changes in portions of basophil granules and the occurrence in the cytoplasm of membrane bound

vesicles were found in control CBH reactions injected with PBS (Fig. 7) and at antigen injected CBH sites (Figs 8–10). In this study there was no evidence that this represented a form of degranulation. When degranulation was induced and followed over 20 min there was no increase in these hypothesized aspects of piece-meal degranulation, nor was there a link with the basophil degranulation by exocytosis which was progressively observed at CBH reactions. Coalescence of occasional vacuoles of 'haloed' granules in basophils at control CBH sites (Fig. 7) could indicate the occurrence of low grade anaphylactic degranulation in the absence of reinjected antigen.

Anaphylactic mediator release at CBH sites. Our results strongly suggest that the phenomenon we have called cutaneous basophil anaphylaxis depends on local basophil accumulations. However, CBH reaction sites differ from normal skin in several other aspects. Vasodilation, infiltration of other cells, and the presence of a variety of inflammatory mediators might make these sites more sensitive to vascular permeability producing factors which are routinely released when antigen is injected into normal skin of immunized animals. For example, it has been demonstrated by Williams & Morley (1973) that certain prostaglandins augment the vasopermeability changes produced by a given dose of histamine in normal guinea-pig skin. We have examined this question by comparing the effect of histamine and compound 48/80 at CBH skin compared to normal skin and have found no augmented reactivity; in fact, CBH sites had a significantly reduced response compared to normal skin (Fig. 5). This cannot be due to problems of diffusion since KLH causes augmented permeability at CBH sites and KLH has a very large molecular weight compared to histamine. Hyporeactivity to Compound 48/80, a substance known to release mediators from guinea-pig mast cells, means that basophils at CBH sites are relatively unresponsive to this substance. The reason for the reduced response to mediators at CBH sites is unknown. It could be due to desensitization of local vessels (Schwartz, Askenase & Gershon, 1977), perhaps indicating prior ongoing release of vasoactive factors at delayed reactions, as is suggested from other studies (Gershon, Askenase & Gershon, 1975; Voisin & Toullet, 1960; Morley & Williams, 1975; Colvin & Dvorak, 1975).

The biological significance of mediator release by basophils in CBH. Basophil degranulation occurs far more frequently in CBH responses of guinea-pigs to live penetrating schistosome larvae (Askenase, Hayden & Higashi, 1976a) than we have observed 24 or 48 h after injection of a conventional delayed skin test dose of a soluble protein antigen like KLH. Some of the Schistosomule associated degranulation is clearly by exocytosis, but some involves partial but extensive dissolution of many individual basophil granules, without accompanying signs of exocytosis and with the occurrence of numerous cytoplasmic vesicles (Askenase, 1977, fig. 18). This may be akin to piece-meal degranulation noted by Dvorak *et al.* (1973) at responses to tumour cells.

Thus, changes consistent with *bothanaphylactic* and piece-meal degranulation of basophils have been noted in guinea-pig reactions to parasites and to tumour cells (Dvorak *et al.*, 1973; Huxtable & Rothwell, 1976). One hypothesis to draw these various findings together is as follows: Basophils are first specifically attracted to tissue sites via chemotactic factors resulting from the interaction of specific antibody and/or sensitized T lymphocytes with surface antigens of multicellular parasites or tumour cells. Subsequent release of antigens by parasites or tumour cells might then interact with antibodies coating newly arrived basophils and cause degranulation and release of vasoactive mediators. Alternatively, new pulses of antigen might stimulate nearby lymphocytes to release factors governing mediator release from basophils (Thueson, Speck & Grant, 1977). In this context, it is important to note that a conventional delayed hypersensitivity skin test with a soluble protein antigen may provide only the initial attracting antigens. Subsequent pulses of antigen, as we have injected, may be a more relevant model of the dynamic events occurring at sites of clinical host-parasite or host-tumour interactions. What effect piece-meal or anaphylactic release of basophil mediators might have on parasites or tumour cells remains to be determined. However, it is noteworthy that when large accumulations of basophils and mast cells occur at immune inflammatory reactions to parasites, release of vasoamines from these cells often accompanies the immune expulsion of the organisms (Askenase, 1977). It seems to us that this type of protective anaphylactic reaction may provide a selective evolutionary advantage and that clinical diseases of immediate anaphylactic hypersensitivity may be an aberration of this response.

Since CBH-type responses are largely restricted to the skin and gastrointestinal tract, the ability to recruit basophils to sites of delayed hypersensitivity *and then* to trigger CBA may be a mechanism for providing augmented local anaphylaxis to function in the expulsion of some parasites.

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REFERENCES

- ASKENASE P.W. (1976) Cutaneous basophil hypersensitivity uncovered in the cell transfer of classical tuberculin hypersensitivity. *J. Immunol.* **117**, 741.
- ASKENASE P.W. (1977) Role of basophils, mast cells, and vasoamines in hypersensitivity reactions with a delayed time course. *Prog Allergy*, **23**, 199.
- ASKENASE P.W., HAYDEN B.J. & HIGASHI G.I. (1976a) Cutaneous basophil hypersensitivity and inhibited macrophage migration in guinea pigs with schistosomiasis. *Clin. exp. Immunol.* **23**, 318.
- ASKENASE P.W., HAYNES J.D. & HAYDEN B.J. (1976b) Antibody mediated basophil accumulations in cutaneous hypersensitivity reactions of guinea pigs. *J. Immunol.* **117**, 1721.
- ASKENASE P.W., HAYNES J.D., TAUBEN D. & DEBERNARDO R. (1975) Specific basophil hypersensitivity induced by skin testing and transferred using immune serum. *Nature (Lond.)*, **256**, 52.
- CHAN B.S.T. (1972) Ultrastructural changes in guinea-pig bone marrow basophils during anaphylaxis. *Immunology*, **23**, 215.
- COLVIN R.B. & DVORAK H.F. (1975) Role of the clotting system in cell-mediated hypersensitivity. II. Kinetics of fibrinogen/fibrin accumulation and vascular permeability changes in tuberculin and cutaneous basophil hypersensitivity reactions. *J. Immunol.* **114** 377.
- DVORAK A.M., DVORAK H.F. & KARNOVSKY M.J. (1972) Uptake of horse-radish peroxidase by guinea pig basophilic leukocytes. *Lab. Invest.* **26**, 27.
- DVORAK A.M., MIHM M.C. JR. & DVORAK H.F. (1976a) Degranulation of basophilic leukocytes in allergic contact dermatitis reactions in man. *J. Immunol.* **116**, 687.
- DVORAK H.F., COLVIN R.B. & CHURCHILL W.H. (1975) Specificity of basophils and lymphocytes in cutaneous basophil hypersensitivity. *J. Immunol.* **114**, 507.

- DVORAK H.F. & DVORAK A.M. (1975) Basophilic leukocytes: structure, function and role in disease. *Clin. Haematol.* **4**, 641.
- DVORAK H.F., DVORAK A.M. & CHURCHILL W.H. (1973) Immunologic rejection of diethylnitrosamine-induced hepatomas in strain 2 guinea pigs. Participation of basophilic leukocytes and macrophage aggregates. *J. exp. Med.* **137**, 751.
- DVORAK H.F., DVORAK A.M., SIMPSON B.A., RICHESON H.B., LESKOWITZ S. & KARNOVSKY M.J. (1970) Cutaneous basophil hypersensitivity. II. A light and electron microscopic description. *J. exp. Med.* **132**, 558.
- DVORAK H.F., MIHM M.C. JR. & DVORAK A.M. (1976b) Morphology of delayed-type hypersensitivity reactions in man. *J. Invest. Dermatol.* **67**, 391.
- DVORAK H.F., SIMPSON B.A., BAST R.C. & LESKOWITZ S. (1971) Cutaneous basophil hypersensitivity. III. Participation of the basophil in hypersensitivity to antigen-antibody complexes, delayed hypersensitivity and contact allergy. Passive transfer. *J. Immunol.* **107**, 138.
- EVANS R., PENCE H., KAPLAN H. & ROCKLIN R.E. (1976) The effect of immunotherapy on humoral and cellular responses in ragweed hayfever. *J. clin. Invest.* **57**, 1378.
- GERSHON R.K., ASKENASE P.W. & GERSON M. (1975) Requirement for vaso-active amines in the production of the skin reactions of delayed-type hypersensitivity. *J. exp. Med.* **142**, 732.
- HASTIE R. (1971) The antigen-induced degranulation of basophil leukocytes from atopic subjects studied by phase-contrast microscopy. *Clin. exp. Immunol.* **8**, 45.
- HASTIE R., LEVY D. & WEISS D. (1976) The antigen-induced degranulation of basophil leukocytes from atopic subjects, studied by electron microscopy. *Lab. Invest.* **36**, 173.
- HAYNES J.D. & ASKENASE P.W. (1977) Cutaneous basophil responses in neonatal guinea pigs: Active immunization; hapten specific transfer with small amounts of serum; and preferential elicitation with phytohemagglutinin skin testing. *J. Immunol.* **118**, 1063.
- HUXTABLE C.R. & ROTHWELL T.L.W. (1976) Studies on the responses of basophil and eosinophil leukocytes and mast cells to the nematode *Trichostrongylus colubriformis*. III. Ultrastructural changes in basophils and eosinophils at the site of infection. *Aust. J. exp. Biol. med. Sci.* **53**, 437.
- LICHTENSTEIN L.M., GILLESPIE E. & BOURNE H. (1973) Studies on the biochemical mechanisms of IgE-mediated histamine release. In: *The Biological Role of the Immunoglobulin E System* (Ed. by K. Ishizaka and D. Dayton) U.S. Government Printing Office, Washington, D.C.
- MORLEY J. & WILLIAMS T.J. (1975) Characterization of delayed hypersensitivity by measurement of local changes in vascular permeability: the place of the Jones-Mote reaction. *Agents and Actions*, **4**, 227.
- NORMAN P.S. & LICHTENSTEIN L.M. (1973) Capacity of purified antigen and whole pollen extracts to release histamine from leukocytes of hay fever patients. *J. Allergy clin. Immunol.* **52**, 94.
- ORANGE R.P. (1973) Immunopharmacological aspects of bronchial asthma. *Clin. Allergy*, **3**, 521.
- SCHWARTZ A., ASKENASE P.W. & GERSON R.K. (1977) The effect of locally injected vasoactive amines on the elicitation of delayed-type hypersensitivity. *J. Immunol.* **118**, 159.
- SOTER N.A. & AUSTEN K.F. (1976) The diversity of mast cell derived mediators: Implications for acute, subacute and chronic cutaneous inflammatory disorders. *J. Invest. Dermatol.* **67**, 313.
- STADECKER M. & LESKOWITZ S. (1974) The cutaneous basophil response to mitogens. *J. Immunol.* **113**, 496.
- TERRY R.W., BAINTON D.F. & FARQUHAR M. (1969) Formation and structure of specific granules in basophilic leukocytes of the guinea pig. *Lab. Invest.* **21**, 65.
- THEUSON D.O., SPECK L. & GRANT J.A. (1977) Histamine-releasing activity produced by human mononuclear cells. *Fed. Proc.* **36**, 1300.
- VOISIN G.A. & TOULLET F. (1960) Modifications of capillary permeability in immunological reactions mediated through cells. In: *Ciba Foundation Symposium of Cellular Aspects of Immunity* (Ed. by G.E.W. Wolstenholme and M. O'Connor) p. 373, J. & A. Churchill, Ltd., London.
- WILLIAMS T.J. & MORLEY J. (1973) Prostaglandins as potentiators of increased vascular permeability in inflammation. *Nature (Lond.)*, **246**, 215.