

The Cellular Dermal Infiltrate in Experimental Immediate Type Cutaneous Hypersensitivity

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A previously developed guinea pig model for the study of the dermal inflammatory cell infiltrate of allergic, toxic, and irritant reactions was adapted to the study of the immediate intradermal reaction to ovalbumin. Comparison of qualitative and quantitative counts of infiltrating cells at three levels in the dermis showed that counting 20 subepidermal fields starting from the injection point of the allergen gave reliable figures. The reaction showed microscopically two phases. The first was of rapid onset and characterized by a high proportion of neutrophils, unlike the picture seen in the previously studied (allergic and toxic) reaction types. In the second phase, which can be termed "late phase" reaction, mononuclear cells and basophil granulocytes predominated. The late phase of the reaction bears similarities to the delayed allergic contact reaction at the same timepoint in that the response was rich in basophils. There were, however, other differences; e.g. eosinophils and neutrophils were more common. **Key words:** skin reaction; guinea pig; inflammatory cells; differential counting;

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The strength of cutaneous inflammatory reactions is classically recorded by the assessment of the degree of erythema and oedema. Histologically the degree of the infiltrate and the cell types present can also be used to assess the intensity of the reaction. Animal models make possible the chronological study of reactions and thus give important background information on the efferent phase of cutaneous reactions.

In a guinea pig model, we have established a cell counting method in which a classical "3"+ system of naked eye assessment of an inflammatory reaction is complemented by qualitative and quantitative counting of the dermal infiltrating cells in various reaction types (1). In normal guinea pig skin there is always a light infiltrate of mononuclear cells (lymphocytes and monocytes) and granulocytes (1). Plastic embedding, thin sectioning and optimal staining are required to achieve the optimal resolution necessary to differentiate neutrophil, eosinophil and basophil granulocytes from each other and from mast cells (1–3). In our method, mast cells are counted in order to ensure their positive differentiation from other granulated cells (neutrophil, eosinophil and basophil granulocytes) which migrate as a part of the cellular traffic to the reaction site. The number of cells that are present in normal skin in the same animal are subtracted from the number of cells in the test site to give a "cell response". Initially the model involved allergic delayed hypersensitivity reactions and toxic reactions to single open applications of test substances (1, 4, 5). Differences in the type and number of inflammatory cells in the dermis have been observed

between these two reactions (5). We have also studied the contact reactions produced by multiple applications of weak toxic agents, a reaction type we have referred to as "irritant" (6). The immediate humoral (reaginic) reactions to ovalbumin (7), in which IgE (and in guinea pig subclasses of IgG) antibodies mediate anaphylactic degranulation of mast cells, exemplify another pathogenetic form of reaction. The cellular infiltrate in this reaction attenuates as the distance from the site of injection increases (7, 8). Cells are also seen to extend deeper into the dermis than in the previously studied reaction types.

The aim of the present paper was to examine, using control materials from previously reported studies (7, 8), the reliability of the original counting method and two modifications of the method and to compare the features of the ovalbumin reaction with previously studied reaction types (4–6).

MATERIALS AND METHODS

Animals

Female Duncan Hartley guinea pigs of 250–300 g body weight, raised in standardized conditions, were used. The present study utilized histological material from previously reported studies (7, 8).

Sensitization

Sensitization for the immediate allergic reaction was performed 14 days prior to testing. Ten µg ovalbumin was injected intraperitoneally together with 100 mg Al(OH)₃ (9). This model was developed to optimize reaginic antibodies as manifested by bronchial anaphylaxis and passive cutaneous anaphylaxis.

Testing

The skin of the flanks was shaved with an electric razor on the day prior to testing. At testing, 10 µg of ovalbumin was injected intradermally using an Agla Micrometer Syringe Dutfit (Wellcome Reagents Ltd., Bechenham, UK).

Macroscopic assessment

Since in guinea pig skin the clear distinction of the oedematous area from the erythematous area is difficult, the diameters of the roughly circular reactions were measured in two perpendicular directions. A mean area was calculated, assuming a circular shape.

Biopsies

For cell counting in the immediate type reactions, a 4-mm biopsy was taken, with one edge on the site of injection. Normal skin was taken from an area not affected by testing.

Histology

The biopsies were fixed in 10% neutral phosphate-buffered formalin, embedded in glycol methacrylate and polyethylene glycol (Sorrvall Embedding Kit, DuPont, USA), cut in 3-µ sections (Hisorange LKB, Sweden) and stained with May-Grünwald-Giemsa (BDH, Axel Johnson, Malmö, Sweden).

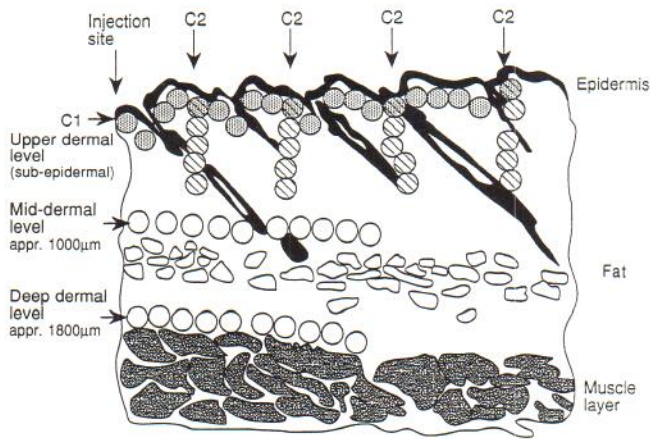


Fig. 1. Schematic representation of a histological section of guinea pig skin (original magnification 40x). All circles \odot \otimes \ominus designate one high-power field 180 μ in diameter. Counting was started from the injection site at all levels. In the upper dermal level, the "C1" method is represented by the symbol \odot and the "C2" by the symbol \otimes . The microscopic localization of the mid- and deep dermal levels is shown.

The cells counted

Our aim was to count "blood-derived" cells, especially granulocytes. A prerequisite for this is the use of plastic embedding and thin sectioning. The cells have been differentiated on the basis of their nuclear structure and granular morphology.

Neutrophil granulocytes. These cells have markedly segmented nuclei and fine granules, which are often only faintly seen in the cytoplasm.

Eosinophil granulocytes. These cells are mononuclear or have bilobed nuclei, and the numerous cytoplasmic granules are brick red in colour.

Basophil granulocytes. The nuclei of these cells are more segmented than those of eosinophils but less so than those of neutrophils. The granules are large and stain in a dark blue or violet colour.

Mast cells. These cells have a single, large, often oval nucleus and a large cytoplasm with relatively diffuse limits. They may appear star-shaped or dendritic. Granules are numerous, small and violet in colour.

Mononuclear cells. At the time of the experiments, guinea pig (monoclonal) markers were not available. In addition, the use of an immunohistochemical technique would have precluded plastic embedding. Lymphocytes are expected to be the dominant mononuclear cell type. They are recognized by their size and their nuclear mass/cytoplasm ratio. Monocytes (with typical peripheral blood morphology) are rarely seen. Mast cells are mononuclear but easily distinguishable because of their granules. Resident cells such as vascular endothelial cells, fibroblasts and histiocytes are also mononuclear cells and sometimes difficult to differentiate from monocytes.

Cell response

The cell response is the number of cells in tested skin minus the number of cells in normal skin. This procedure compensates for normally infiltrating cells and other resident cells in the dermis and gives a qualitative and quantitative assessment of the cell traffic in and out of the reaction area.

Counting of the dermal inflammatory cell infiltrate

Fig. 1 illustrates three levels in the guinea pig skin designated *upper*, *mid-* and *deep dermal levels*. The original counting method "C" was developed for quantitating the cellular response of cutaneous delayed hypersensitivity contact reactions in which the cellular infiltrate is situated directly beneath the dermoepidermal junction. Using a 100 x oil immersion lens, 20 arbitrarily chosen high-power fields were counted. Care was taken to obtain fields that had no hair follicles – this necessitated analysis of more than one section of the biopsy.

Since the inflammatory cell infiltrate in the immediate reaction dif-

fers from the previous reactions studied, some modifications of the original counting method were undertaken. In the first modified counting method "C1", 20 consecutive fields below the dermoepidermal junction were counted starting at the injection site. Any artifactual or necrotic areas in conjunction to the injection site and hair follicles were excluded. The first clear area underneath the excluded site was then counted.

In the second modified counting method "C2", the 20 counted fields comprised 4 groups of 5 high-power fields perpendicular to the dermoepidermal junction and 5 high-power fields apart. Fig. 1 illustrates the above-mentioned counting methods in the upper, mid- and deep dermal levels.

In order to examine the cell infiltrate even deeper in the skin, cell counting was performed at the mid-dermal level 6 high-power view-fields (approximately 1,000 μ) under the dermoepidermal junction and in the deep dermal level, approximately 1,800 μ under the dermoepidermal junction (the area immediately above the muscle layer).

The counting in the deeper levels was started on the same side of the section as the injection site. Ten consecutive fields were counted in mid- and deep dermis (the biopsy was often conical in shape and thus no wider than approximately 10 fields at these levels).

Experimental design

Macroscopic assessment and dermal inflammatory cell counting using the three counting methods, C, C1 and C2 will be presented for one animal group ($n = 4$). The results of dermal inflammatory cell infiltrate counting using the modified method (C1) will be presented for these animals and two further groups, which were controls in other experiments, as a pooled material ($n = 13$).

Statistical analysis

The results of the cell counting were statistically analysed by Student's paired *t*-test and unpaired *t*-test. In addition, pooled material has been compared using a method previously described (5), in which the allergic reaction to oxazolone at 24 h has been considered the "model reaction".

RESULTS

The total area of the hypersensitivity reaction to ovalbumin for the original group ($n = 4$) is shown in Fig. 2. Although redness and oedema appear rapidly, the reaction does not reach its maximum until 12 h.

Low-power microscopic examination

Examination of the reaction sites showed the tissue at the point of injection to be sometimes necrotic. The cellular infiltrate was seen to attenuate with increasing distance from the injection site.

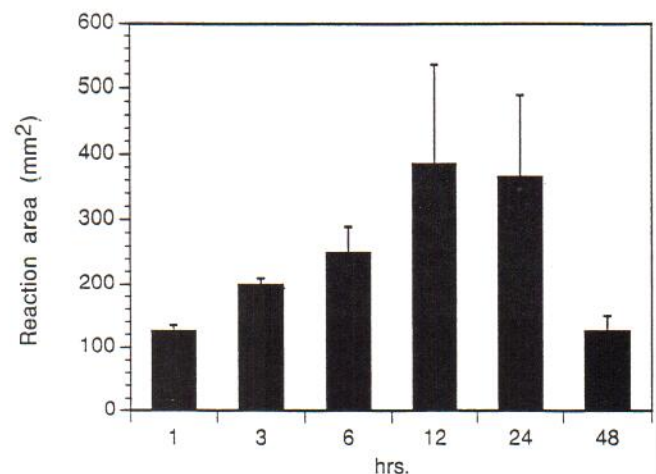


Fig. 2. Total reaction area in guinea pigs sensitized to ovalbumin.

Table I. The cellular response (test count minus normal skin count) after intradermal injection of ovalbumin in sensitized animals

A -(minus) sign before the numbers indicates that the number of cells at the test site was lower than the number found in normal skin.

	MONO	MAST	BASO	EOS	NEUTRO	TOTAL
1 h	-0.70	-0.34	-0.01	0.47	2.58	1.99
3 h	-0.50	-0.29	0.07	0.95	3.45	3.67
6 h	1.25	-0.31	0.15	1.88	2.71	5.69
12 h	5.14	-0.30	1.17	1.43	1.95	9.39
24 h	6.77	-0.24	2.05	2.58	0.83	11.99
48 h	6.43	-0.33	5.11	3.30	0.38	14.89

At 1 h, the number of infiltrating cells was small and they were evenly distributed between upper mid- and deep dermal levels. An increase in mononuclear cells (mainly lymphocytes) in the upper dermal level was seen in the 3-, 6- and 12-h reaction sites. In mid- and deep dermal levels, polymorphonuclear granulocytes were evident in and around the vessels. The 24- and 48-h reactions showed a profusion of mononuclear cells and granulocytes in the upper dermal level, and little or no accumulation of cells in and around the deeper vessels.

High power microscopic examination

Comparison of the different counting methods in the upper dermal levels was performed (Fig. 1). Test reactions at 1, 3, 6, 12, 24 and 48 h were examined. A differential count consisting of mononuclear cells, basophils, eosinophils, neutrophils, and mast cells was performed for each time period. Figures were similar for the three counting methods, but the total cell count was somewhat higher in the modified counting method (C1). We

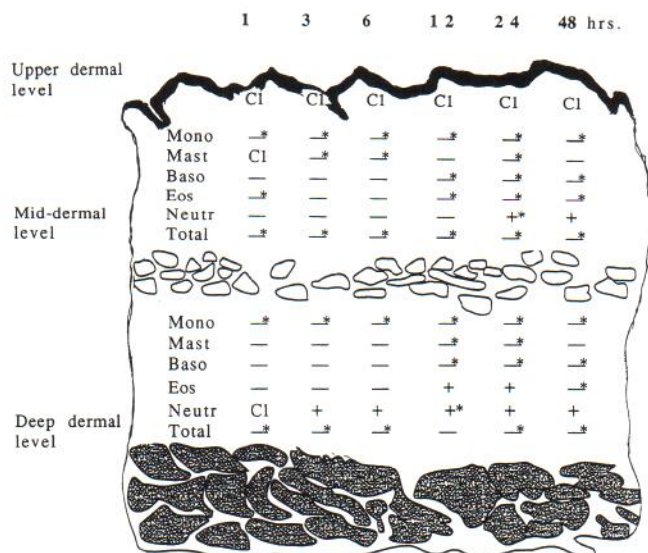


Fig. 3. Inflammatory cell counting at 3 levels in the dermis of sensitized guinea pigs ($n = 13$) after intradermal injections of ovalbumin. The upper dermal (subepidermal) level is taken as the reference level (C1) and compared to the infiltrates at the mid and deep dermal levels at different times. Similar levels (within 5% of C1) are designated C1. - or + designate trends in cell numbers; -* or +* designate significant changes between the levels ($P < 0.01$).

therefore chose this method as representative of the upper dermal level.

The results for the pooled control group ($n = 13$) using the modified (C1) method for upper dermal counting are presented in Table I. Figures are shown as the "cell response". The mononuclear cells (mainly lymphocytes) show a decrease during the early phase of the reaction and reach a peak only after 24 h (12-48 h, $p < 0.01$). Mast cells are reduced in number during the entire experimental period (3-48 h, $p < 0.01$) and the basophils, whilst reduced in number after 1 h, increase in the later stage of the reaction to reach a peak at 48 h (12-48 h, $p < 0.01$). Neutrophil counts peak at 3 h, being statistically significantly increased at 1 h ($p < 0.01$). Eosinophils, although increased in number at all time points, are highest later in the reaction with statistically significant values between 12 and 48 h ($p < 0.01$).

The validity of relying on the upper dermal counts was checked by counting at the mid-dermal and deep dermal levels. Counts at the different levels also illustrate dynamic aspects of inflammatory cell traffic in these reactions. In Fig. 3 the upper dermal count was taken as a reference level. Total counts at the mid- and deep dermal levels were always less than in the upper dermal count. Basophils and mast cells were less numerous in the mid- and deep dermal counts and were in fact rarely seen. Variation in the amount of cells in the different animals was noted, but not in the cell differential. The most pronounced difference between the upper dermal count and that in mid- and deep dermal levels was that neutrophils were more numerous. They were seen predominantly perivascularly in increased numbers in the mid-dermal level late in the reaction and at the deep dermal level at all time points from 3 h. Eosinophils were seen in increased numbers in the deep dermal level at 12 and 24 h.

The chronology of the cellular inflammatory response already presented in Table I can be highlighted by expressing results as a differential (Table IIa). Early in the reaction (up to about 6 h), neutrophils and eosinophils predominate (85 and 31% of the infiltrating cells, respectively). A second stage (from 12 h) is comprised chiefly of basophils and mononuclear cells, predominantly lymphocytes (33 and 55%, respectively). Mast cell numbers were decreased for the duration of the experiment (1-48 h).

In order to facilitate discussion of the present results and a comparison with previously studied reaction types, we have presented the inflammatory cell response seen in the allergic, toxic and irritant reaction types (5, 6) in Table IIb. We have previously shown that there are statistically significant differences in absolute cell counts between these pooled allergic and toxic reaction materials (5). Using the same method, the 24-h reaction to ovalbumin is statistically different from the 24-h allergic reaction to oxazolone for all cell types. The same was true when the 24-h reaction to ovalbumin was compared to the 24-h reaction to croton oil (5).

DISCUSSION

One of the most important features of dermal inflammatory cell counting is the selection of representative fields. In our view, the modification (C1 count in the upper dermis) of our previous method has proved reliable, when compared with other counts in which the selection of viewing fields is technically more diffi-

Table II(a). The inflammatory cell response in the type I reaction to ovalbumin expressed as %

A decrease in cell numbers is designated as "loss".

	MONO	MAST	BASO	EOS	NEUTRO
(a) Type I Reaction (ovalbumin n = 13)					
1 h	loss	loss	loss	5.0	85.0
3 h	loss	loss	1.6	21.3	77.2
6 h	21.0	loss	2.5	31.3	45.2
12 h	53.0	loss	12.0	14.8	20.1
24 h	55.0	loss	16.8	21.1	6.8
48 h	42.2	loss	33.6	21.7	2.5

Table II (b). Cellular response in fully developed allergic, toxic (from ref. 3) and irritant reactions (from ref. 4) are shown to illustrate the varying spectrum of the inflammatory cell infiltrate in other studied reaction types

(b) Previously studied reaction types					
Allergic (oxazolone 24 h n = 52)	64.5	1.2	29.8	3.8	0.6
Toxic (crotonoil 24 h n = 53)	85.1	3.1	8.6	3.3	1.6
Irritant (2% NaLS 3 × 3 n = 76)	84.0	1.9	2.5	3.0	8.5

cult. Some differences in the cellular infiltrate at deeper levels in the dermis do of course exist – infiltrates are in general less pronounced and neutrophil granulocytes (and to a lesser extent eosinophils) are seen in greater numbers. The total cell count is, however, always largest in the upper dermis.

The reaction to ovalbumin shows two waves of cellular infiltrate. In the first phase, neutrophils and eosinophils predominate and there is a marked loss of mast cells. This is a pathogenetically hardly surprising feature of the immediate reaction, implying, as it does, degranulation. The mast cell loss continues throughout the reaction. Eosinophils are also present in both the early and the late phase at levels much higher than those seen in the previously studied reaction types. The mononuclear cell count was reduced at 1 and 3 h. The significance of this finding is uncertain but may have to do with cellular redistribution. From 6 h, mononuclear cell numbers successively increased but did not reach the levels seen in the previously studied reaction types.

In the late phase of the reaction, basophils and mononuclear cells (mainly lymphocytes) predominated. The histological demonstration of basophils is difficult, for which reason their role has not always been commented in previous studies of the immediate type reaction. Cutaneous basophil-rich reactions have been seen in delayed (cell-mediated) immune responses to many antigens, including contact allergens in both guinea-pigs and humans. Many antigens induce antibody formation as well as cell-mediated immunity (10, 11). The concept of "cutaneous basophil hypersensitivity" (CBH) was introduced by Dvorak et al. (2) and has been the subject of many studies (10, 12). Cutaneous basophil response of delayed type reactions can for

instance be passively transferred by lymph node cells (10) and by immune serum in guinea pigs (3, 11).

In the present study, basophil numbers decreased at 1 h, which may indicate that resident cells of this type have anaphylactic capability. After 1 h basophils begin to accumulate in the skin; at 48 they constitute more than 20% of the cellular response (Table IIb). This level is similar to that seen in an allergic delayed reaction. Anaphylactic degranulation does not seem to be a part of the basophils' role at this later stage. They can perhaps instead serve as auxiliary effector cells, "non-specifically" recruited by actively sensitized T cells, B cells and their products (13).

The question is whether the second stage of the ovalbumin reaction represents a separately acquired allergic reaction of delayed CBH type, or a progression of the original immediate reaction (late phase response). It seems most likely that the second cellular phase can be termed "late phase reaction" as seen previously in cutaneous reactions caused by various agents (8, 14, 15). This is seen in both allergic and non-allergic contact reactions and must be considered a non-specific inflammatory or reparative consequence of the original immediate reaction.

Can the dermal inflammatory cell counting be used to type guinea pig cutaneous inflammatory reaction of *unknown* pathogenesis, for instance in the case of dubious reactions in predictive testing protocols? Some observations which arise from the presentation in Table II may be of use. Mast cell numbers in previously studied reaction types have always increased in guinea pigs (see Table IIb). Therefore, a pronounced decrease in mast cell numbers in a reaction at any time point may indicate an anaphylactic mechanism. The presence of eosinophils in high proportions for the duration of the reaction and an early heavy predominance of neutrophils are other features which, coupled to naked eye observation of a rapid-onset reaction, may be useful criteria for an immediate type pathogenetic mechanism. Extrapolation of conclusions to human studies must be undertaken with caution. It is, however, probable that a late phase cellular infiltrate is to be expected in human immediate type reactions. Caution should therefore be exercised in diagnosing simultaneous immediate type and delayed type sensitization based on the presence of a late histological inflammatory infiltrate.

The present paper shows how chronological, light microscopic study of the inflammatory cell infiltrate can give qualitative and quantitative information, which can be used to illustrate dynamic and pathogenetic aspects of cutaneous inflammation. Inflammatory cells have variable roles in different cutaneous inflammatory reactions. Animal models can, correctly used, fill gaps in our knowledge and give information which contributes to better design of subsequent human studies.

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