

MEASUREMENT AND DIFFERENTIATION OF THE CELLULAR INFILTRATE IN EXPERIMENTAL ALLERGIC CONTACT DERMATITIS

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Abstract. We describe a quantitative method for the grading of contact allergic reactions in guinea pigs. These reactions are characterized by marked cellular infiltration, and the method is based on total and differential counting of cells in the upper corium. A varying and objectively gradable increase in mononuclear and basophil polymorphonuclear cells was found. In naked-eye-positive reactions this increase was highly significant 24, 48, and 72 hours after epicutaneous application of dinitrochlorobenzene (DNCB). The degree of cellular infiltration reflects aspects of a cell-mediated immune response other than the visible reaction ordinarily made use of. The method can be used to study how systemically or topically administered drugs affect cellular features in contact allergy.

Key words: Contact allergy; Cellular infiltrate; Differential counting; Mononuclears; Polymorphonuclears

The quantitation of cell-mediated immunity in the individual subject has been fraught with uncertainty. A number of highly interesting *in vitro* models have been introduced, but none of the methods developed enables us to measure the individual degree of hypersensitivity. The intensity of cutaneous reactions *in vivo* remains the most valuable sign. This is also the case in contact allergy, where epicutaneous test reactions are at the same time reflection of the allergic dermatitis in man and certain animals. The guinea pig remains the traditional experimental animal, since the naked-eye picture and histology of the skin reaction closely resemble those in human allergic contact dermatitis. The classical way to assess the test reaction is to estimate the degree of redness and oedema (21), i.e. the visible signs of inflammation. The same principle has since been modified by others, and is still a standard method (12, 22). Another useful technique is to measure the increase in ear swelling of animals after application of the test substance (2). However,

all these methods are based on an inflammatory sign caused by vascular dilatation and extravasation.

Microscopic study of an inflammatory process will also reveal cellular infiltration. Histological changes in contact hypersensitivity reaction have been studied in detail (10, 11, 15, and others). Epidermal spongiosis, vascular dilatation in the corium, and cellular infiltration initially in the corium and later also in the epidermis are the most prominent features. Studies on the different cell types participating in contact hypersensitivity reaction in guinea pigs and in man have been performed by several workers (3, 5, 6, 8, 9, 16, 17, 19, 23, 26, 28). On the basis of variations in mononuclear cell infiltrate in the epidermis in test lesions, a useful method of grading the contact hypersensitivity reactions was developed: the number of infiltrating lymphoid cells is expressed as a percentage of all cells in the epidermis (13, 14). This method is very time-consuming, however.

Various histopathological techniques have been tried in order to find a simpler method for total and differential counting of cells infiltrating the upper dermis in test lesions and normal skin.

In the present study guinea pigs were sensitized to DNCB and tested with non-toxic doses of the same antigen. The aim has been to quantitate the mononuclear and polymorphonuclear cells infiltrating the upper dermis in test lesions and to utilize any response by particular cells in order to grade the test lesion and consequently also the degree of hypersensitivity.

MATERIAL AND METHODS

Animals

Thirty female albino guinea pigs (Dunkin-Hartley strain) were used; they weighed approximately 300 g at the time of sensitization. The animals were kept at a temperature of 22-25°C in separate cages, and were fed on standard pel-

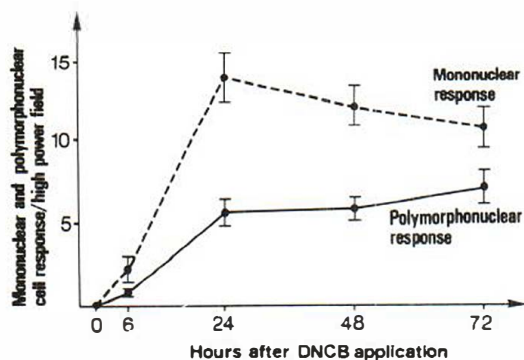


Fig. 1. Quantitation of mononuclear and polymorphonuclear cell infiltration in the upper corium of tested skin of DNCB-sensitized guinea pigs. The cellular response is given as the cell count in tested skin minus that in normal skin. Each point represents the mean of all tests on 30 animals. All animals were tested 6, 24, 48, and 72 hours before biopsy.

lets (Astra-Ewos AB, Sweden). The experiments were carried out at several sessions, 5 animals (average) being used on each occasion.

Sensitization

The neck and shoulder regions were shaved with an electric razor. The animals were then sensitized epicutaneously with 20% dinitrochlorobenzene (DNCB) in acetone. 0.02 ml was applied to an area of about 1 cm², first on one side and on the following day on the other. A local toxic reaction with redness and crusts occurred in every animal.

Testing

About 14 days after the sensitization both flanks were shaved, at least 2 hours before testing. 0.02 ml of 0.2% DNCB dissolved in acetone or alcohol was applied to a skin surface of 2 cm², the test dose being 20 µg DNCB/cm². This dose had previously been proved atoxic in several non-sensitized animals. To study the development of the reaction, single test doses were applied to different areas of the flanks 72, 48, 24, and 6 hours before the assessment. After naked-eye inspection the animals were killed and a biopsy specimen (diameter 4 mm) was taken from each test spot and from untested skin.

Naked-eye inspection

The reactions were graded as follows:

- 0 = No change, or uncertain reaction.
- +
- ++ = Redness, with slightly palpable induration.
- +++ = Redness and obvious swelling.

Histological technique

The biopsies were fixed in 10% neutral phosphate-buffered formalin and embedded in glycol methacrylate and polyethylene-glycol (JB-4 Plastic Embedding Kit[®],

Polysciences, USA). Sections 3 µm thick were cut. Harris's haematoxylin-eosin was used routinely, and May-Grünwald-Giemsa (24) was used for further classification of the polymorphonuclear cells. Some specimens were fixed in 5% neutral phosphate-buffered glutaraldehyde and post-fixed with osmium tetroxide (7).

Cell counting in the corium

Several widely-spaced sections were cut from each specimen. The mononuclear and polymorphonuclear cells were counted in 20 high-power fields in the upper corium, just beneath the epidermis (1000×, oil-immersion objective). The result was defined as the number of cells in one high-power field. The cell count in the normal, intact corium varies to some degree among different individuals. The increase in the test areas could therefore be calculated by reducing the cell count by the number of cells in normal skin of the same animal. We have called the difference the *mononuclear cell response* or *polymorphonuclear cell response* in the corium. With the aid of this we were able to compare the individuals and to eliminate possible influences of unspecific factors such as shaving.

Classification of cells in the corium

Hair follicles, fibroblasts, vascular endothelial cells, and cells in vascular lumina were not counted. Cells which were impossible to classify were also excluded.

Mononuclear cells. As a rule all these cells were included in a single group called 'mononuclears', but in some cases they were subgrouped as follows:

1. Small mononuclear cells. (a) Small lymphocyte. Cell with a small, round, dark-stained, homogeneous nucleus and scanty cytoplasm. (b) Medium-sized lymphocyte. As above, but the nucleus is slightly larger and as a rule less darkly stained.

2. Large mononuclear cells. Larger than the above. The nucleus is often oval, slightly irregular, and lightly stained. This subgroup probably comprises large lymphocytes, monocytes, histiocytes (macrophages) and mast cells when haematoxylin-eosin staining is used.

Polymorphonuclear cells. Cells with a rod-shaped or segmented nucleus and granules in the cytoplasm were included in this group. In order to identify different classes

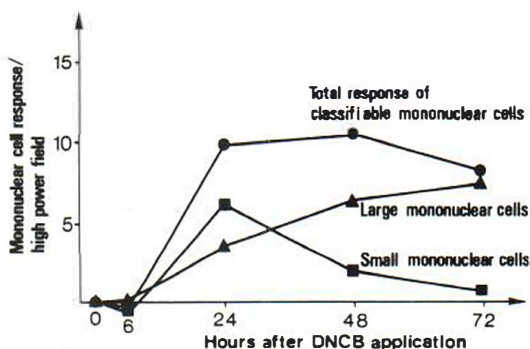


Fig. 2. The behaviour of small and large mononuclear cells in test lesions after application of DNCB in 5 sensitized animals.

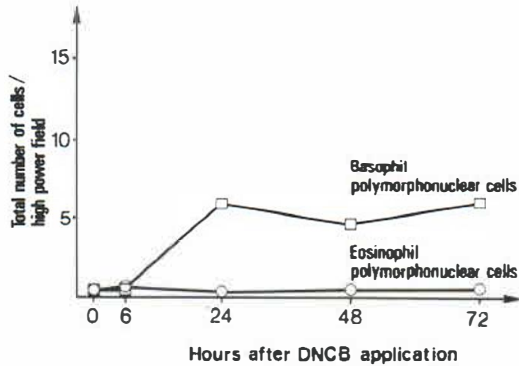


Fig. 3. The total number of basophil and eosinophil cells per field in the upper corium in normal skin and test reactions in DNCB-sensitized guinea pigs (means for 4 animals). The neutrophil count, not recorded here, was less than 1 cell per 10 fields in all sections.

of polymorphonuclears some sections were stained with May-Grünwald-Giemsa. The classification was made on the basis of the appearance of the nucleus and granules in the cytoplasm (25) and staining properties with May-Grünwald-Giemsa (24).

1. Neutrophil. Nucleus often markedly segmented. Small, scattered, red granules in the cytoplasm.

2. Eosinophil. Nucleus less markedly segmented. Granules larger than those in neutrophils, brick red, and closely packed.

3. Basophil. Nucleus less markedly segmented than that of neutrophil. Granules particularly large and stained dark violet or blue (Fig. 6).

Haematoxylin-eosin stained the granules various shades of red in all polymorphonuclears, but the difference in the size of granules described above was obvious.

Mast cells. These mononuclear cells were identified with May-Grünwald-Giemsa. The nucleus is oval or round and lightly stained. The small granules seen in the cytoplasm are stained violet.

Statistical method

Student's *t*-test was used.

RESULTS

Each high-power field of normal intact corium just below the epidermis contained about 25 mononuclears and one polymorphonuclear cell. The count varies to some degree among different individuals. In Fig. 1 the mononuclear and polymorphonuclear response to epicutaneous application of a non-toxic dose of DNCB in sensitized animals is shown. Each point on the curve represents the mean for test lesions in 30 animals. There was a significant increase ($p < 0.05$) in mononuclear cells 6 hours after the application of DNCB. Be-

tween 6 and 24 hours the increase continued ($p < 0.001$). Subsequently no statistically significant change occurred within the observation period. The polymorphonuclears also increased between 0 and 6 hours ($p < 0.001$) and between 6 and 24 hours ($p < 0.001$), after which there was no significant change. The increase in both mononuclear and polymorphonuclear cells was thus highly significant in the 24, 48, and 72 hour tests, on comparison with normal skin.

The behaviour of small and large mononuclears after DNCB application is illustrated in Fig. 2. Certain mononuclears cannot be classified, owing to the angle of section, and so there is some loss of cells here. Each point on the curve represents the mean for 5 animals. Small mononuclears tended to reach a maximum at 24 hours and then decrease. In contrast, there was a more sustained increase in large mononuclears, which were therefore considerably more numerous than the small mononuclears at 72 hours.

The intact corium contains about 1 basophil or 1 eosinophil per field (Fig. 3, means for 4 animals), whereas neutrophils are extremely sparse. When DNCB is applied to skin of a previously sensitized animal the increase in the total number of polymorphonuclear cells (Fig. 3 and Fig. 1) is due to basophils, absolute number of eosinophils remaining constant. Neutrophils were rare in the tested skin. No change in the mast cell count during the test reaction (compared with normal skin) could be seen in these 4 animals.

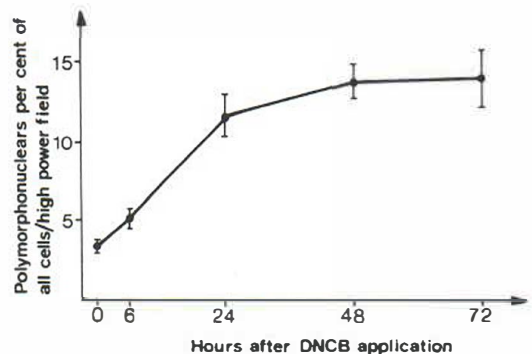


Fig. 4. The proportion of polymorphonuclear cells as a percentage of all cells per field ($100 \times P/S$. P = polymorphonuclear cells. S = polymorphonuclear + mononuclear cells) in tested skin of 20 DNCB-sensitized guinea pigs.

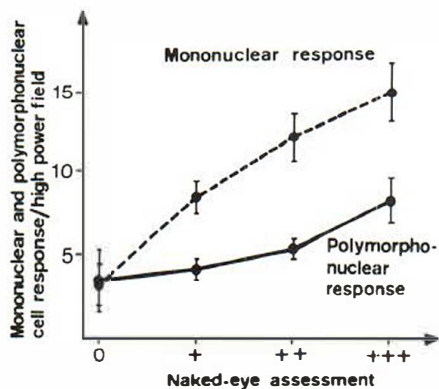


Fig. 5. Mononuclear and polymorphonuclear cell response in 30 DNCB-sensitized guinea pigs. Comparison with the naked-eye assessment is made regardless of the time (6, 24, 48 or 72 hours) after the application of dinitrochlorobenzene at which the reading was carried out.

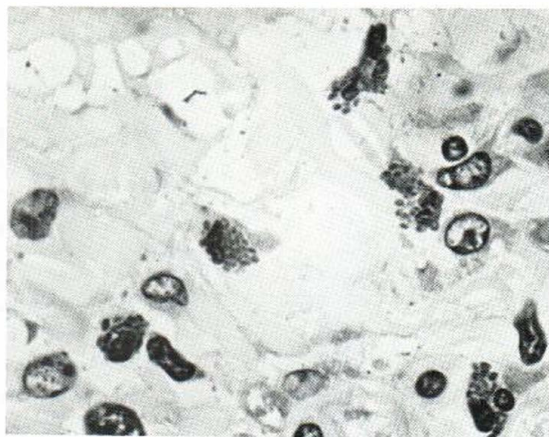


Fig. 6. Cellular infiltrate in the upper corium in a 48 hour DNCB-test reaction. Mononuclear cells and several basophils.

The proportion of polymorphonuclear cells per field ($100 \times P/S$, P =polymorphonuclear cells, S =mononuclear + polymorphonuclear cells) increases during the first 6 hours ($p < 0.01$) and shows a further rise between 6 and 24 hours ($p < 0.001$) (Fig. 4). The increase in polymorphonuclears is due almost entirely to increase in basophils (Fig. 3), which means that the basophil count increases both absolutely and relatively during the first 24 hours of the development of the allergic contact reaction, after which it remains fairly constant. After 24 hours a large number of basophil granules are seen in the test reactions. The state which appears to have established itself may possibly be explained as a balance between lysis of cells and concomitant cellular invasion.

Most of the test reactions were graded + or ++ on naked-eye assessment, and such reactions showed a variable but convincing increase in cellularity. Fig. 5 shows a comparison between macroscopic assessment and the mononuclear and polymorphonuclear cell infiltration. An increase in cellularity was also seen in some naked-eye-negative tests.

DISCUSSION

In the light of previous experience we have sought to work out a reliable, reproducible standard method for absolute and differential counting of cells infiltrating the upper dermis, that is, the region showing the most marked degree of cellular infiltra-

tion in allergic contact reactions. Classification of cells by light microscopy of histological sections is considerably more intricate than assessment of cells in smears or imprints. The appearance of the cells is affected by the method of fixation and by the fact that the cells are fixed at different phases of movement as they pass through the collagenous tissue. The appearance of the cells may also be influenced by the site and angle at which the nucleus and cytoplasm are cut by the microtome. The staining properties also vary greatly. Because cellular infiltration is often non-uniform, comprehensive counts have to be made over wide areas of the reaction in order to obtain reliable figures. This is particularly necessary when the degree of cellular infiltration is to be used to grade the intensity of the reaction.

The techniques of formalin fixation and methacrylate embedding used by us are simple and give good quality sections. Counts can therefore be made over wide areas and in several sections of the same test area. May-Grünwald-Giemsa staining makes possible identification of basophil, eosinophil, and neutrophil leukocytes in addition to mononuclear cells. Using serial sections and Harris's haematoxylin-eosin stain we have succeeded in differentiating the various polymorphonuclear leukocytes, mainly on the basis of differences in size and shape of cytoplasm granules, even when colour differences have been slight.

The classification of mononuclear cells in sections has been the subject of a number of investigations (5, 20, 28). In the study now presented we give

the total mononuclear cell count: all attempts at subgrouping will result in the exclusion of certain cells for technical reasons, and the distinctions between different cells will be inexact. Nevertheless, we have in some animals attempted to make a distinction between large and small mononuclear cells.

Our method of counting cells in intact and tested skin shows that the corium normally contains a small 'pool' of mononuclear and polymorphonuclear cells that probably enter the tissue via the vessels. This normal cellular infiltration shows slight individual variations, and is probably influenced by even quite mild unspecific factors such as shaving, light, and heat. By subtracting the number of cells found in the normal skin from the test reaction cell count in the same animal a closer measure of the number of cells 'attracted' by the allergic reaction can be obtained. This also results in greater certainty when comparing different individual animals and different series of animals.

The total mononuclear cell infiltration reached its maximum within 24 hours of applying DNCB, and subsequently remained fairly constant throughout the 72-hour observation period. The predominant cell type was the mononuclear, irrespective of whether the total cellular infiltration varied between different test reactions in different animals. Our figures confirm impressions earlier reported (10, 11). It was also found that small mononuclears reach their maximum within 24 hours and subsequently decrease, and that there is a more sustained increase in the number of large mononuclears, which cells remain numerous throughout the observation period. Turk et al. (28) and Braun-Falco et al. (5) report in histochemical studies on contact hypersensitivity that at the peak of the reaction most of the mononuclear cells are lymphocytes.

In the past the polymorphonuclear cells have perhaps received less attention than the mononuclears in connection with contact hypersensitivity. An increased basophil count has been reported in studies using skin window technique (29), however, and in the cytology of blister fluid (4, 18). Since 1970 systematic investigations have been carried out into the incidence of basophil leukocytes in different types of delayed hypersensitivity reactions, most with the aid of a special histomorphological technique and electron microscopy (3, 7, 8, 9, 16, 17, 19, 23), and it has been plainly shown that there is a marked increase

in the incidence of basophil leukocytes in the cellular infiltrate in contact allergic reactions.

The intact dermis normally contains small numbers of basophils and eosinophils in individually varying proportions, whereas neutrophils are scanty. When DNCB is applied to the skin of a previously sensitized animal the increase in polymorphonuclear count that takes place is due to an increase in basophil cells, the absolute eosinophil count remaining unchanged. In the corium, on the other hand, eosinophils have been observed in contact hypersensitivity to another antigen (15, 16), in 're-test' reactions (1, 6), and in immunological unresponsiveness to DNCB (27).

The nature of delayed hypersensitivity reactions remains obscure, and a number of hypotheses have been put forward. In these reactions mononuclear cells predominate, and it is accepted that the lymphocyte has a primary function. Detailed investigations have been carried out on the interaction between lymphocytes, monocytes, macrophages, basophils, and Langerhans cells. Our studies confirm the common occurrence of the close 'cell-to-cell relationship', especially in the immediate vicinity of the epidermis.

Until the pathogenesis of contact hypersensitivity is fully explained, the choice of method for assessing the intensity of contact allergic reactions will remain arbitrary. Naked-eye inspection is based on the degree of the vascular reaction, and in doubtful cases leaves much scope for subjective interpretation. Histological studies with cell counting are more objective, and from the point of view of pathogenicity may be more interesting: there is evidence that the primary allergic reaction is to be sought in one or several of the cellular components of the inflammatory reaction.

We have outlined the mononuclear and polymorphonuclear cell response in the upper dermis at different times after the application of DNCB to sensitized guinea pigs and have found a varying and objectively gradable increase in both mononuclear and basophil cells. The counting of these infiltrating cells in test reactions constitutes a quantitative method of grading the intensity of test reactions and also the degree of individual hypersensitivity. This cellular response reflects aspects of a cell-mediated immune reaction other than the visible reaction that is ordinarily made use of. In a series of investigations we have employed this standard model to obtain an idea of how factors

such as cytostatic drugs and systemically or topically administered steroids affect cellular features in contact allergy.

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