Immunology 1983 **49** 415

A study of cells present in lymph draining from a contact allergic reaction in pigs sensitized to DNFB

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Accepted for publication 6 January 1983

Summary. Pigs were skin-painted with the contact sensitizing agent 1-fluoro 2,4 dinitrobenzene (DNFB) and lymph cells coming from the site were collected. Half the animals were sensitive to DNFB and half were normal controls. Special attention was paid to cells belonging to the veiled cell series. At 20 hr after DNFB application, some blast cells—on morphological grounds belonging to the veiled cell series—could be observed in the lymph of presensitized animals. But the most predominant finding in these pigs was that, approximately 30 hr after painting, the total cell output began to increase, reaching 10 times the normal level at 70 hr. The increase in the output of lymphoblasts was the most marked, suggesting peripheral sensitization. Such changes were not observed in normal animals. Here the most striking alterations were seen in the veiled cells. Some of these cells were moving ponderously about, apparently in pursuit of other lymph-borne cells, and this activity resulted in the formation of large cellular aggregates. Since it is known that veiled cells are involved in antigen handling, this behaviour probably facilitates the induction of the T-cell immune response. By contrast, the veiled cells from presensitized animals behaved in a more normal manner and the proportion found in aggregates was only briefly increased. In these animals lymphocytes were seen to interact with veiled cells in a manner reminiscent of peripolesis, apparently recognizing antigenic signals on the surface of the veiled cells. It is discussed that this might result in deletion of the antigen-presenting veiled cells, thus controlling the magnitude of the immune response.

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0019-2805/83/0700-0415$02.00
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INTRODUCTION

Contact sensitivity reactions are characterized by reddening and oedema of the skin with mononuclear cell infiltration starting 6 hr after painting. In these reactions epidermal and dermal Langerhans cells are surrounded by lymphocytes and show signs of degeneration (Silberberg, Baer & Rosenthal, 1974). Occasionally a Langerhans-like cell is seen in a dermal lymphatic (Silberberg *et al.*, 1975). This suggests that contact sensitizing agents induce a delayed response which is directed against the sensitizing agent presented on the surface of the Langerhans cell. The immuno-competent lymphocytes probably recognize hapten in combination with Ia-like structures, which are also present on the surface of the Langerhans cell (Klareskog *et al.*, 1977; Rowden, Lewis & Sullivan, 1977). Large veiled cells, which closely resemble Langerhans cells, have been found in lymphatics

draining the skin (Drexhage *et al.*, 1979; Spry *et al.*, 1980) and similar cells are also present in afferent gut lymph of rat (McPherson & Steer, 1980). In this lymph they may have been derived from Langerhans-like cells in Peyer's patches (Wilders *et al.*, 1983). The traffic of cells present in lymphatics draining a contact allergic skin reaction to oxazolone has been investigated by Hall (1980) in sheep; an increased output of neutrophil leucocytes was found, lasting for about 48 hr. Thereafter the cell output remained elevated, but the majority of cells were small lymphocytes accompanied by increased numbers of lymphoblasts. It appeared that in this species the reaction was first of the Arthus type, though a cell-mediated component may have been present during the later phases of the reaction. No data were given on lymph-borne Langerhans-like cells separately, though a considerable increase was reported in the traphic of macrophages and macrophage-like cells. In pigs sensitized to 1-fluoro 2,4 dinitrobenzene (DNFB) typical delayed-type skin reactions with mononuclear cell infiltration were obtained 14 days after sensitization; circulating antibodies to dinitrophenol (DNP) could not be demonstrated until 3 weeks after painting (McFarlin & Balfour, 1973). It seemed of interest to investigate in these animals the cellular kinetics in the draining lymphatics in particular at day 14, a time when the cell-mediated component would be predominant and antibodies which would induce Arthus reactivity would not be detectable in the circulation.

MATERIALS AND METHODS

Animals

Large white pigs from the Chelmwood Herd (Søeberg *et al.*, 1978) weighing 90–140 kg were used as experimental animals.

Skin painting with DNFB and lymphatic cannulation

The pigs were anaesthetized and the superficial lymphatics on the inner aspect of one front leg were dissected and Portex cannulae inserted as described (Søeberg *et al.*, 1978; Drexhage *et al.*, 1980). The animals were allowed to regain consciousness and lymph collection was started as soon as the flow was satisfactory.

The volume of lymph was measured at hourly intervals and the total number of cells per ml counted, as were the number of free veiled cells, the number of veiled cells in aggregates and the total number of cells per aggregate. Millipore membrane preparations of the lymph cells were also made at hourly intervals (Drexhage *et al.*, 1979). After 2–3 hr, 100 μl of a freshly prepared solution of 10% DNFB in a 1 : 1 (v/v) mixture of acetone and dimethylsulphoxide (DMSO) were applied to the inner aspect of the carpal region of each front leg, and lymph collection continued for periods up to 70 hr. Five animals were used in this experiment.

Another five animals had been presensitized to DNFB a fortnight before by applying 50 μl of a freshly prepared solution of 10% DNFB to each flank. Lymphatic cannulation and skin painting were performed as in normal pigs.

Time-lapse cinematography of living lymph cells

Lymph was collected from normal and sensitized animals at various times after the application of DNFB and time-lapse cinematography was carried out as described (Drexhage *et al.*, 1979).

Membrane collection of prefixed lymph cells

Samples of lymph were taken from both groups of animals before DNFB application and at hourly intervals after painting. The samples were fixed, collected on Millipore membranes and stained as described (Drexhage *et al.*, 1979). Differential counts of the various cell types were carried out. The output per hour of the different types of cells was calculated from the differential counts and the total cell output per hour.

Preparation of lymph cells for transmission electron-microscopy

Lymph was collected directly into a mixture of cold glutaraldehyde and osmium tetroxide as described (Hirsch & Fedorko, 1968), and the cells were pelleted and embedded in low viscosity epoxy resin. Thin sections were prepared on an LKB-Ultratome III and examined in a Philips 300 electron microscope.

RESULTS

Normal animals

The total cell output from normal skin on the inner aspect of the carpal region of normal pigs averaged 18×10^5 (range 11–25) cells per hour, of which 13×10^5 (range 7–17·5) were lymphocytes, $1{\cdot}5 \times 10^5$ (range 0·9–2·8) were atypical mononuclear cells (a heterogenous group which included large granular lympho-

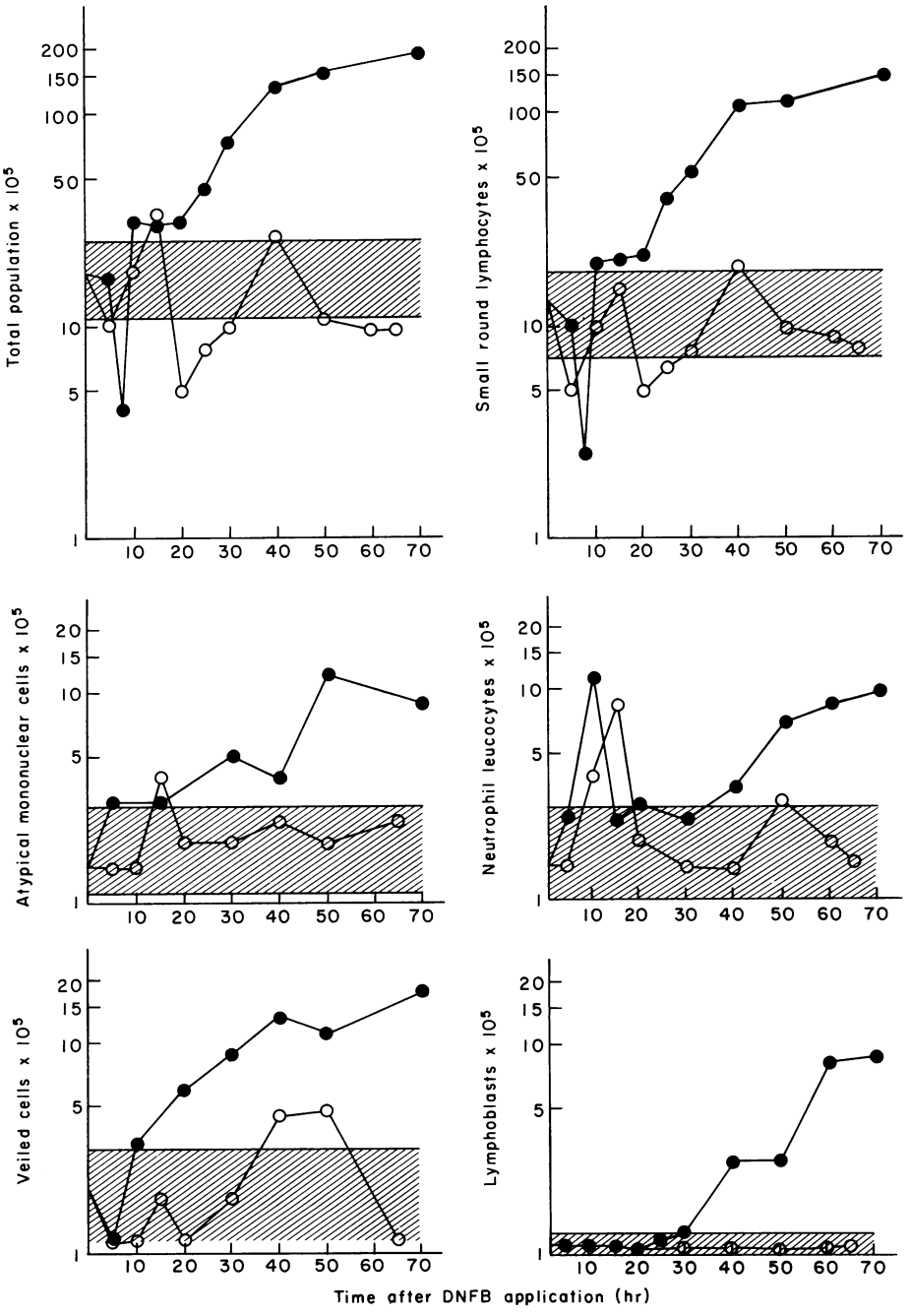

Figure 1. Total output of cells per hour, output of small round lymphocytes, atypical mononuclear cells, neutrophil leucocytes, veiled cells and lymphoblasts, in lymph from skin of foreleg after DNFB application. (○) Normal pigs; (●) pigs pre-sensitized to DNFN; hatched area, output before DNFB application.

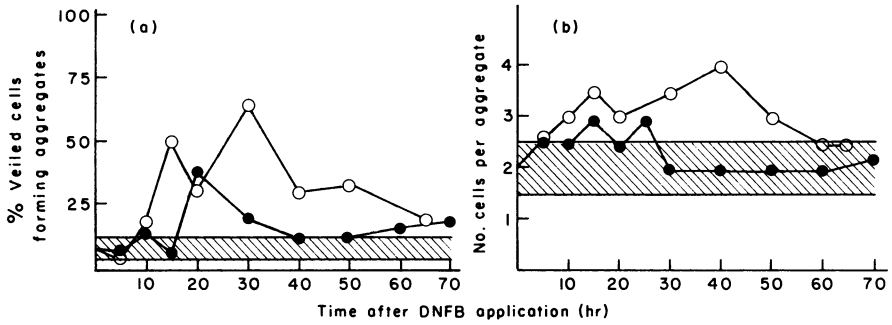

Figure 2. (a) Percentage of veiled cells forming aggregates, (b) number of cells per aggregate, in lymph from skin of foreleg after DNFB application. (○) Normal pigs; (●) pigs pre-sensitized to DNFB; hatched area, aggregate formation before DNFB application.

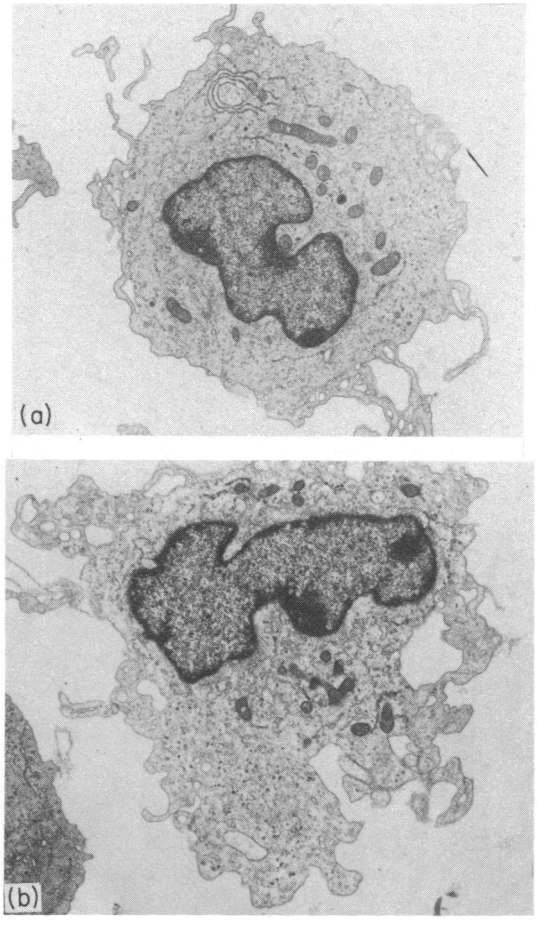

Figure 3. Electron micrographs of veiled cells in lymph from normal skin of foreleg. (a) This cell has several thin cytoplasmic extensions which contain no organelles; the nucleus is convoluted and in an eccentric position; there is a little RER and a few polyribosomes, some microfilaments and mitochondria and many small vesicles (magnification × 5780). (b) This cell has an irregular outline with broad projections and short thick processes; there are many polyribosomes, but fewer microfilaments and mitochondria (magnification × 5780).

cytes, monocytes and other cells not yet fully characterized), and 2×10^5 (range 1–3) were veiled cells, $1{\cdot}5 \times 10^5$ (range 0·0–3·0) cells were neutrophils, and $0{\cdot}2 \times 10^5$ (range 0·0–0·4) were lymphoblasts. During the first 5 hr after painting, the total cell output fell to approximately half the average value, due largely to a fall in cell concentration. At 15 hr it increased to almost twice the average, declined again to a third of the average, and from 30 hr onwards was more or less in the normal range with some fluctuation. The output of lymphocytes followed the same pattern, except that there was no increase in output above the normal range at 15 hr. The increase at that time was accounted for mainly by a five-fold increase in the output of neutrophil leucocytes and a smaller increase in the output of atypical mononuclear cells. The output of veiled cells was reduced to one-third of the average value at 5 hr and from 15 hr onwards followed the usual pattern, with a slight increase after 40 hr. The output of blast cells was not significantly altered during the whole period of observation (Fig. 1).

In normal lymph, about 7% of the veiled cells were found to be in cellular aggregates with other cells; this proportion was increased seven-fold at 15 hr, then decreased slightly and there was a second nine-fold increase at 30 hr, followed by a gradual decline (Fig. 2a). The number of cells in each aggregate was also increased, the largest aggregates being found at 40 hr (Fig. 2b).

The behaviour of living lymph-borne cells, coming

from the site of painting, was studied by time-lapse cinematography and the results of these investigations have already been reported (Drexhage *et al.*, 1979). In summary, they showed that normal veiled cells continually extrude boat-shaped veils, by means of which they engulf fluid, apparently sampling the surrounding medium; they do not seem to move in a directional manner. On the other hand, veiled cells, coming from a site of DNFB painting, extrude very large fan-shaped veils, they develop a peculiar wasp-waisted outline and stop engulfing fluid; these cells are active in contacting other lymph-borne cells, forming large cellular clusters.

Scanning and transmission electron microscopic studies of lymph-borne cells coming from normal skin and after the application of DNFB have also been reported (Drexhage *et al.*, 1979). Further investigation showed that the veiled cells in normal lymph form an even more heterogenous group than was originally supposed. The majority possess the characteristic long thin cytoplasmic extensions and some of these cells contain phagolysosomes (Fig. 3a). But there are also veiled cells with broad angular projections as well as veils (Fig. 3b), which may contain several medium-sized vacuoles; some of these have thin outer walls, others have thick walls and may have been formed by

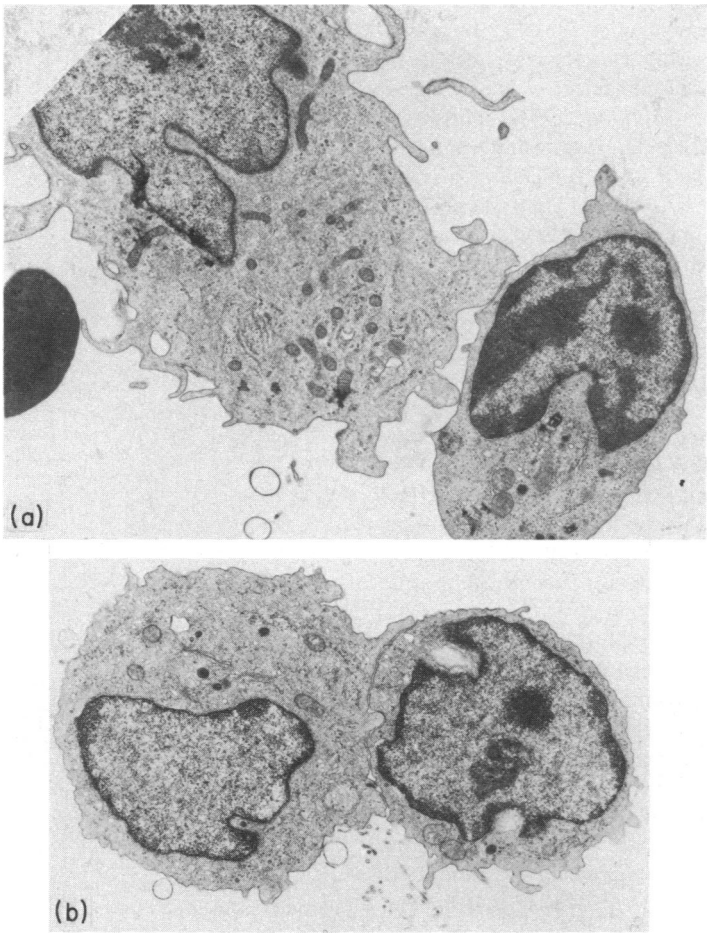

Figure 4. Electron micrographs of cells in lymph from skin of pre-sensitized pig 12 hr after DNFB application. (a) Large elongated veiled-type cell with an eccentric nucleus; the cell processes are in contact with a monocyte-like cell containing dense bodies (magnification ×6800). (b) Small lymphocyte in contact with another lymphoid cell, probably a monocyte or a large granular lymphocyte; the area of contact between the cells extends over a considerable part of both cell surfaces (magnification ×6800).

fusion of several short processes. Such cells contain larger numbers of polyribosomes but fewer microfilaments, mitochondria and small vesicles. The morphology of these cells is very reminiscent of that of the macrophage-like cells described by Morris (1968) in lymphatics draining the testis, the ovary, the thyroid, the liver and the kidney of sheep.

Sensitized animals

The total cell output in lymph coming from the contact allergic reaction was reduced to a quarter of the average value 7 hr after painting, then increased to approximately twice the average level at 10 hr, and from 25 hr onwards increased steadily to reach 10 times the average value at 70 hr. This large increase in cell output was mainly accounted for by an increased output of lymphocytes, with a comparable increase in the output of atypical mononuclear cells and veiled cells. The output of neutrophil leucocytes followed a somewhat different pattern. During the first 5 hr it was in the normal range, with a sharp increase to seven times the average value at 10 hr; by 15 hr it was again in the normal range and remained at a low level until 50 hr, then started to increase again, reaching seven times the average level at 70 hr. The output of blast cells was increased two-fold at 10 hr and from 30 hr onwards continued to increase, reaching even 50 times the average value at 70 hr (Fig. 1).

The percentage of veiled cells found in aggregates with other cells was in the normal range during the first 15 hr after painting, then increased five-fold at 20 hr, returning to the normal range at 40 hr (Fig. 2a); but these peak values were always lower than those found in non-sensitized pigs. The number of cells per aggregate was slightly increased during the first 30 hr after painting (Fig. 2b).

The behaviour of living lymph-borne cells was again recorded by time-lapse cinematography. In contrast to the situation in normal animals skin painted with DNFB, the lymphocytes themselves seemed to be active in contacting veiled cells. This phenomenon was first observed 7 hr after painting. Some of the adherent lymphocytic cells were seen to extend and withdraw long processes over the surface of the veiled cell in a vigorous stroking movement, which was repeated many times. At this time the veiled cells had a circular outline and continued to extrude boat-shaped veils and engulf fluid as in normal lymph, but a small proportion resembled the activated veiled cells seen in lymph from non-sensitized animals. Later the proportion of activated veiled cells increased.

Lymph-borne cells were also collected and processed for electron microscopy. In lymph collected 12 hr after challenge the majority of lymphocytes did not show any obvious ultrastructural changes. These cells and also monocyte-like cells containing dense bodies and many polyribosomes were seen in contact with large non-lymphoid cells. This latter group was very

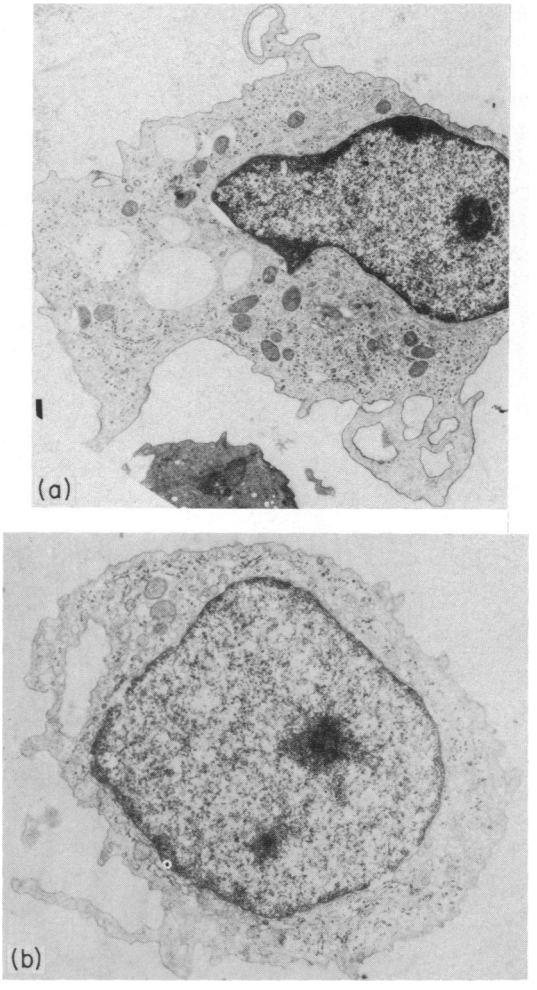

Figure 5. Electron micrograph of cells in lymph from skin of pre-sensitized pig 20 hr after DNFB application. (a) Large non-lymphoid cell with short cytoplasmic extensions, an eccentric open nucleus with nucleolus, and numerous polyribosomes in the outer part of the cytoplasm as well as some RER; the cell seems to be engaged in active protein synthesis (magnification × 5780). (b) Non-lymphoid blast cell with a long cytoplasmic process and vacuole, features reminiscent of the veiled cell lineage; the nucleus is very large, with loose chromatin and a prominent nucleolus (magnification × 5780).

heterogenous, there were large cells which were very much like veiled cells and had an elongated almost rectangular shape, short processes, an eccentrically placed nucleus, small amounts of rough endoplasmic reticulum (RER), a few mitochondria, sparse micro-filaments and many small vesicles (Fig. 4a). But there were also cells which were much smaller, had no processes at all and these cells were very similar to monocytes (Fig. 4a), and the area of contact sometimes extended over a considerable part of the surface with a hint of interdigitating.

At 20–28 hr a few blast-like cells were observed. Some of these had an elongated shape, with short processes, an eccentrically placed nucleus, some perinuclear micro-filaments, abundant cytoplasm with many polyribosomes in the outer part of the cell and several large vacuoles situated at the pole opposite to that occupied by the nucleus (Fig. 5a). Others had a very large open nucleus, a few short processes and scanty cytoplasm containing some polyribosomes (Fig. 5b). Both these cell types are, on histologcial appearance, most likely members of the monocyte/veiled cell series. At 30 hr, the lymph contained large numbers of lymphoctyes of a very uniform type, with a prominent nucleus, fairly abundant electron lucent cytoplasm and some polyribosomes. After 40 hr lymphoblasts began to appear in the lymph in increasing numbers.

DISCUSSION

These findings confirm the observation that in non-sensitized pigs peripheral recruitment and transformation of immunocompetent lymphocytes did not take place to any extent after painting with the contact sensitizing agent DNFB (Drexhage *et al.*, 1979). In sheep, however, it was reported that a first application of oxazolone was followed by a large increase in the traffic of lymphocytes and lymphoblasts in the afferent lymph coming from the site (Hall, 1980). The toxic effects of DNFB may have prevented peripheral sensitization in our experiments, but did not interfere with the process of sensitization since the animals gave typical delayed-type hypersensitivity reactions when challenged 14 days later (McFarlin & Balfour, 1973). In pigs, recruitment of cells responsible for contact reactivity must have taken place in the lymph node or some other distant site.

In sensitized sheep the response to challenge was of the Arthus type, with an increased output of neutrophil leucocytes during the first 48 hr (Hall, 1980). In sensitized pigs there was only a brief increase in neutrophil leucocyte output during this period, and a similar reaction was seen in non-sensitized animals following skin painting. It would seem therefore that the pig is a more suitable model to study contact reactivity since it is not complicated by Arthus reactivity.

In contact allergic reactions in guinea-pigs sensitized to DNFB, the epidermal Langerhans cells at the site of challenge were found to be surrounded by lymphocytes (Silberberg *et al.*, 1974), and this arrangement was interpreted to mean that the lymphocytes had recognized the hapten on the surface of the Langerhans cell. In DNFB-sensitized pigs, lymph-borne lymphocytes coming from the site of challenge during the first 7–12 hr after painting were seen to contact veiled cells.

Since cells coming from the site of DNFB application carry small amounts of DNP (Søeberg *et al.*, 1978), it is likely that the lymphocytes recognize hapten on the surface of the veiled cell as they do on Langerhans cells. These contacts are probably followed by the release of lymphokines and other mediators which are responsible for delayed reactivity, although Hay, Lachmann & Trnka (1973) failed to detect migration inhibition factor (MIF) activity in afferent lymph coming from a tuberculin reaction. Some of the attached lymphocytic cells began stroking the surface of the veiled cell in a manner reminiscent of the phenomenon of peripolesis (Sharp & Burwell, 1960), which is considered a cytotoxic event. In this process, antigen-bearing target cells may be lethally damaged by the attacking lymphocytes (Silberberg *et al.*, 1974) causing deletion of antigen-presenting cells, so that the magnitude of the response is controlled. This cell behaviour in sensitized animals is very different from that observed in the non-sensitized, where cellular aggregate formation by active veiled cells is the predominant feature. This behaviour probably facilitates the induction of the immune response. In sensitized animals only a minority of the veiled cells behaved in this way. It is a matter of conjecture whether the mechanisms responsible for aggregate formation had been partly neutralized in these reactions by the production of a set of cell mediators which influenced veiled cell behaviour and differed from those produced in normal inflammatory responses.

Furthermore, it is well known that in contact reactivity lymphocytes and mononuclear cells are

trapped in the skin, probably in the form of cell clusters around Langerhans cells. This event might also be responsible for the disappearance of cell aggregates in the lymph during the later phases of the reaction, since large cellular clusters cannot enter the draining lymphatics.

There were also interactions between veiled cells, monocytes, monocyte-like cells, large granular lymphocytes and small lymphocytes, suggesting a more complex pattern of immunological events in contact allergic reactivity. The appearance of some blast-like veiled cells 20 hr after challenge in the sensitized animals was unexpected, and it is tempting to speculate that these cells had been stimulated to proliferate or to engage in protein synthesis, and thus may have contributed to the spectrum of mediators released in delayed reactivity. They were never seen in lymph from non-sensitized animals and they might have been induced by mediators released in the early phases of the contact allergic reaction.

Twenty-five to thirty hours after challenge the total cell traffic coming from the reaction site started to increase and continued to do so until the end of the experiment. The output of lymphocytes increased in the same manner, but the output of lymphoblasts in the lymph draining a contact allergic reaction increased very sharply, reaching even 48 times the average level after 70 hr. This enormous output of lymphoblasts very likely represents peripheral sensitization.

ACKNOWLEDGMENTS

The authors wish to thank S. Griffiths for help in the preparation of electron micrographs, J. Clarke and N. Papworth for their help with time-lapse cinematography and W. R. Dolman for careful typing of the manuscript.

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