LOCALIZATION OF ANTIGEN ON LYMPH NODE DENDRITIC CELLS AFTER EXPOSURE TO THE CONTACT SENSITIZER FLUORESCEIN ISOTHIOCYANATE

Functional and Morphological Studies

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Contact sensitivity can be induced in mice receiving injections of cells obtained from the regional lymph nodes after skin painting (1). A variety of cell types have been suggested to be responsible for the transfer of sensitization for delayed-type hypersensitivity (DTH)[1] to contact sensitizers. These include T lymphocytes (2–4), macrophages (5), and Langerhans' cells (LC) (6, 7). Recent studies have shown that small numbers of dendritic cells (DC) isolated from the lymph nodes of mice skin painted 24 h previously with contact sensitizers (8–10) or DC isolated from spleen or lymph nodes and exposed to contact sensitizers in vitro (10, 11) transfer sensitization for DTH to recipient animals. Previously we demonstrated that there was an increase in the number of DC in the lymph nodes 24 h after skin painting mice with FITC, with no significant change in the number of lymphocytes (10). In addition, the antigen was preferentially located on DC, and small numbers of these DC sensitized normal recipients showing that DC both carry and transfer the antigen. Here we examine changes in the numbers and properties of cells isolated from the lymph nodes from 30 min to 6 d after skin painting with FITC. We show that during a 3-d period after skin painting, antigen is located preferentially on DC in draining lymph nodes, and these DC stimulate T cell proliferation in vitro.

Materials and Methods

Mice. CBA mice between 6 and 10 wk old were obtained from the specific pathogen–free unit at the Clinical Research Centre. Mice of the same sex were used within experiments.

Cell Suspensions. Inguinal and axillary lymph nodes were taken from normal mice or mice skin painted 1–6 d previously. Single cell suspensions prepared by pressing the nodes through wire mesh were washed in medium (RPMI 1640, Dutch Modification; Flow Laboratories, Irvine, United Kingdom; with 100 IU/ml of penicillin, 100 μg/ml streptomycin, 10^{-5} M 2-ME, and 7.5% FCS). The cells (5–8 ml) at 5×10^6/ml were layered onto 2 ml of metrizamide (Nygaard, Oslo, Norway; analytical grade, 14.5 g, added to 100 ml of medium) and were centrifuged for 10 min at 600 g. Cells at the interface were collected, washed once, and resuspended in medium. These separated cells from normal mice and

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[1] *Abbreviations used in this paper:* DC, dendritic cells; DTH, delayed-type hypersensitivity; LC, Langerhans' cells.

from mice skin painted up to 2 d previously were >75% DC (8), and were sensitive to lysis with a specific antibody for DC (33D1) plus complement (12). <5% were macrophages that labeled with the F4/80 mAb (13), and the remaining cells were lymphocytes. The purity of DC isolated at the interface obtained from the lymph nodes of mice 3 and 4 d after skin painting was reduced to 50% and the proportion of cells sensitive to Thy-1 + C increased.

Enriched T cells (>90%) were obtained by passage of lymph node cells over nylon-wool columns as described by Julius et al. (14). Enriched B cells (85%) were obtained by disruption of the nylon wool to elute adherent cells and were treated with anti Thy-1.2 and 33D1 + C.

In Vivo Sensitization. FITC (isomer 1; Sigma Chemical Co., Poole, United Kingdom) was dissolved in a 50:50 (vol/vol) acetone/dibutylphthalate (BDH) mixture just before application. Mice were painted on the shaved thorax and abdomen with 0.4 ml of 5 mg/ml of FITC (15).

In Vitro Culture. Cultures (20 μl hanging drops in inverted Terasaki plates, reference 16) contained $2-16 \times 10^4$ T cells and received varying numbers of DC irradiated with 2,500 rad (^{60}Co source). The cultures were pulsed with [^{3}H]thymidine (Amersham International, Amersham, United Kingdom; 2 Ci/mM. 1 μl added per culture to give a final concentration of 1 μg of thymidine/ml) for 2 h and harvested by blotting onto filter discs (16, 17).

The acid-insoluble material was counted using a liquid scintillation counter. Differences in log counts per minute significantly greater than replication variability were calculated using analysis of variance and Student's *t* test (18).

Antibody Depletions. Lysis with antibody plus C was used to deplete cell preparations of DC using 33D1 (12) or of T cells using anti Thy-1.2 (AT83A). Cells at 5×10^5 to 5×10^6/ml were mixed with antibody and rabbit serum as a source of complement (Buxted Rabbit Co., Sussex, United Kingdom) or with C alone as a control for cytotoxicity, and the cells incubated for 60 min at 37°C and washed twice.

Electron Microscopy. Cells were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in osmium tetroxide, dehydrated, and embedded in Spurr Resin. Cells were observed in a Jeol 12.00 EX microscope.

Fluorescence Analysis. Cells were suspended in 0.5 ml PBS containing 1 mM EDTA (0.02% sodium azide, 25 μg propidium iodide) (to identify dead cells), and 2% FCS, and were analysed using a FACS II (Becton Dickinson & Co., Mountain View, CA) with the laser set at 300 mW, 488 nm, and the photomultiplier tube at 750 V, with fluorescence intensity displayed using a logarithmic amplifier. Calculations used the accumulated data from 5×10^4 cells for each preparation. The fluorescence distribution of cells obtained after metrizamide separation of lymph node cells was examined. The DC, sensitive to lysis with 33D1, were large cells (light scatter channel number 27–56) and were considered separately from small cells (light scatter channel number 18–26), which were lymphocytes as shown by immunofluorescence using anti–mouse Thy-1.2 and anti–mouse Ig. For some experiments, highly fluorescent DC and less fluorescent DC were sorted from other cells at a flow rate of 2,000 cells/second.

Results

Time Course of Changes in Cell Numbers in the Lymph Nodes after Skin Painting. The changes in total number of DC and lymphoid cells which could be isolated from the draining lymph node was examined over a period of six days after skin painting. By 30 minutes, $27 \times 10^3 \pm 5 \times 10^3$ DC were counted compared with $20 \times 10^3 \pm 3 \times 10^3$ at time 0, i.e., unpainted mouse (Fig. 1a). The number of DC isolated increased sharply during the first 4 h after painting (45×10^3 DC), and then gradually leveled off. The number of DC isolated peaked on day 2 and had returned to near control levels by day 4 (Fig. 1b). The proportion of DC in the whole lymph node preparations from normal mice and from mice skin painted 24 h previously was also examined by sensitivity to 33D1
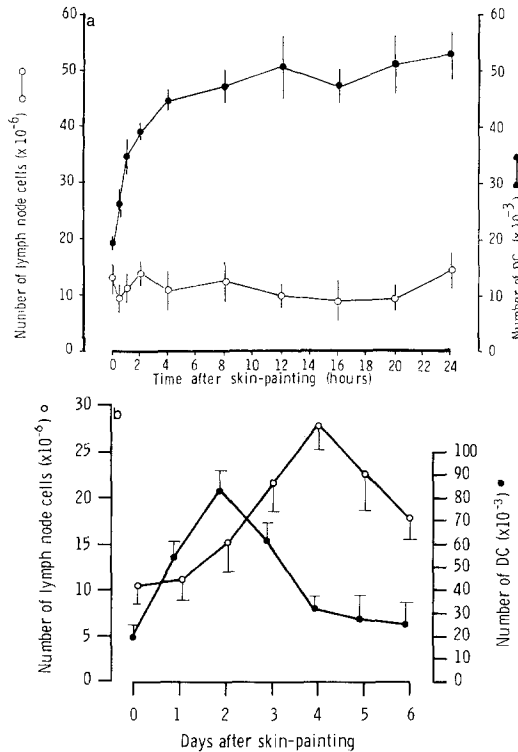
FIGURE 1.   Change in numbers of cells in the lymph node examined at various times after skin painting CBA mice with 2 mg FITC. Results are mean of five experiments, each of which used pooled cells from four mice. (*a*) Change in numbers of lymph node cells from 30 min to 24 h after skin painting. (○) Number of isolated DC; (●) total number of unseparated cells. (*b*) Change in numbers of lymph node cells during 6 d after skin painting. (○) Number of isolated DC; (●) total number of unseparated cells.

+ C and by morphology. ~2 × 10^3 cells were counted in each sample. The number of DC from normal mice was virtually undetectable (<1%), whereas DC from skin-painted mice constituted >5% of the total lymphoid population. The number of large cells passing through metrizamide gradients (pellet cells) was compared between normal mice and mice skin painted 24 h previously, using the FACS. ~25 × 10^4 cells were processed in each sample and there was no change in the proportion of large cells detected. Therefore, the increase in the numbers of DC isolated from the lymph nodes after skin painting reflected a real increase in the number of DC in the lymph node and not a redistribution of cells between the pellet and the interface.

Although the numbers of DC in the lymph nodes increased, the proportion of DC within the node was very small and little change in the total number of cells recovered from the lymph node during the first 24 h after skin painting was detected (Fig. 1*a*). However, there was an increase in the number of lymph node cells that peaked on day 4 and still remained above normal levels on day 6 (Fig. 1*b*).

Fluorescent Cells in Lymph Nodes after Skin Painting with FITC.   The appearance in the draining lymph nodes of fluorescent DC after skin painting with FITC was examined using the FACS. An increase in fluorescence intensity was detectable on DC by 30 min (Fig. 2*a*). By 8 h the distribution of fluorescence on DC was slightly biphasic and this became more apparent at later times (12, 16, and 24 h). The peak in fluorescence intensity occurred at 24 h and had declined by
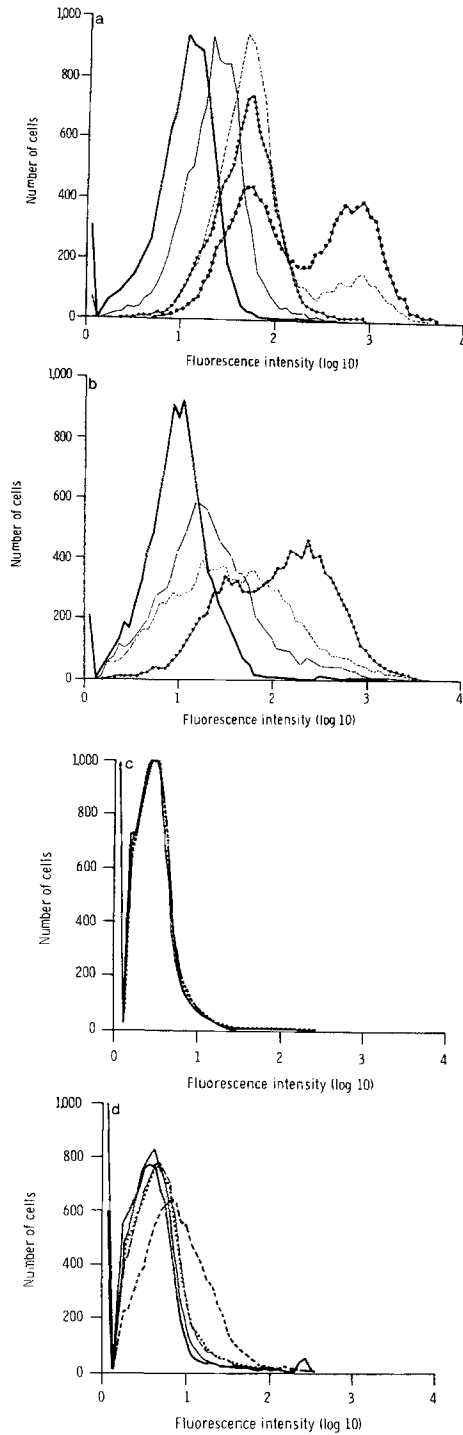
FIGURE 2. Fluorescence on lymph node cells obtained from 30 min to 6 d after skin painting with FITC. (a) (—) Normal DC; (—) DC isolated 30 min after skin painting; (----) DC isolated 2 h after skin painting; (····), DC isolated 8 h after skin painting; (●) DC isolated 24 h after skin painting. (b) (—) Normal DC; (····) DC isolated 1 d after skin painting; (----) DC isolated 3 d after skin painting; (———) DC isolated 6 d after skin painting. Fluorescence on DC isolated on days 2, 4, and 5 was intermediate to that seen here and for clarity is not shown on the graph. (c) (—) Normal T cells; (····) T cells isolated 1 d after skin painting; (—) T cells isolated 3 d after skin painting; (----) T cells isolated 5 d after skin painting. (d) (—) Normal B cells; (—) B cells isolated 1 d after skin painting; (----) B cells isolated 2 d after skin painting; (— —) B cells isolated 3 d after skin-painting; (····) B cells isolated 5 d after skin painting.
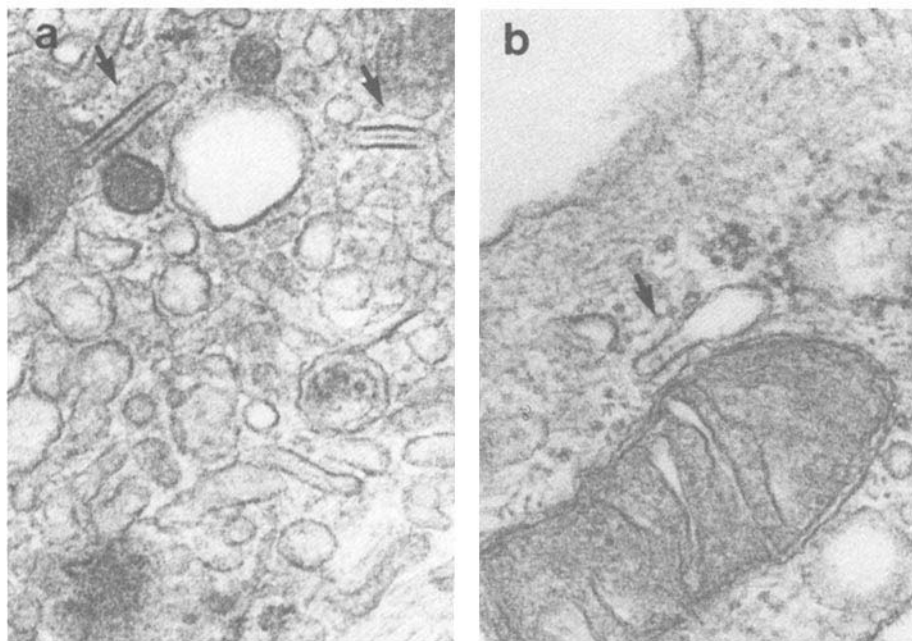
FIGURE 3.   Transmission electron micrographs of lymph node DC from mice 24 h after skin painting with FITC. Arrows indicate Birbeck granules. (a) × 72,000. (b) × 75,000.

48 h (Fig. 2b). High levels of antigen were detected on the DC for up to 3 d, after which the amount of fluorescence fell to near control levels.

The fluorescence profiles for whole lymph node cells (not shown) and separated nylon-wool nonadherent T cells (Fig. 2c) showed no change during the 5 d they were examined. There was an increase in the proportion of cells of a size intermediate between resting lymphocytes and DC, in the whole lymph node cell and T cell preparations, obtained 3 and 4 d after skin painting. These cells were believed to be blasts and stained with an anti-IL-2-R mAb TIB-222. Transient fluorescence was detected on the enriched B cells, either 1, 2, or 3 d after skin painting in repeated experiments (Fig. 2d).

Morphology of DC.   Some DC taken 24 h after skin painting had Birbeck granules (Fig. 3,a and b), which are ultrastructural markers for the epidermal LC. These DC also contained large amounts of rough endoplasmic reticulum (RER), lysosomes (Fig. 4), Golgi, and microfilaments around the nuclear periphery (Fig. 5).

The biphasic distribution of fluorescence on DC seen in the early time course was examined further. DC were isolated from the draining lymph nodes 16 h after skin painting when there was an equal proportion of highly fluorescent and less fluorescent DC. These populations were separated using the FACS, examined by electron microscopy, and their functional capacity was assessed in vitro. Both populations had the characteristics of DC, although the highly fluorescent DC were significantly larger.

DC Exposed to FITC In Vivo Induce Lymphocyte Proliferation.   The level of proliferation of normal T cells (2–16 × 10⁴) was measured after the addition of DC (10³) isolated from mice at various times after skin painting. An increase in
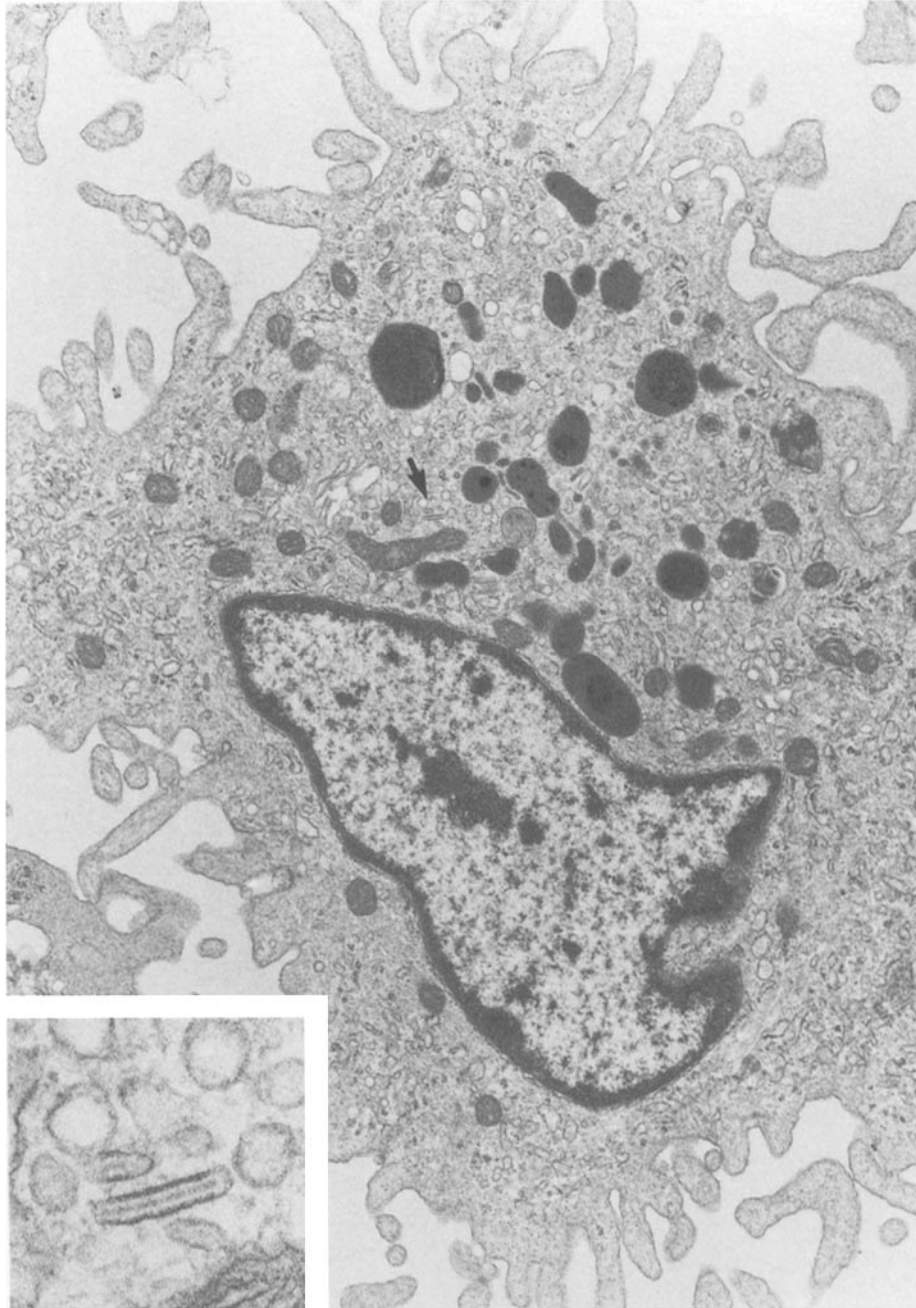
FIGURE 4. High lysosomal content of DC isolated from lymph nodes 24 h after skin painting with FITC (× 13,000). Insert of Birbeck granule (× 85,000).

proliferation was first detected using DC obtained 8 h after skin painting (Fig. 6a), i.e., the time of the appearance of a biphasic distribution in the fluorescence (Fig. 2a).

We examined the functional capacity of DC sorted into highly fluorescent and
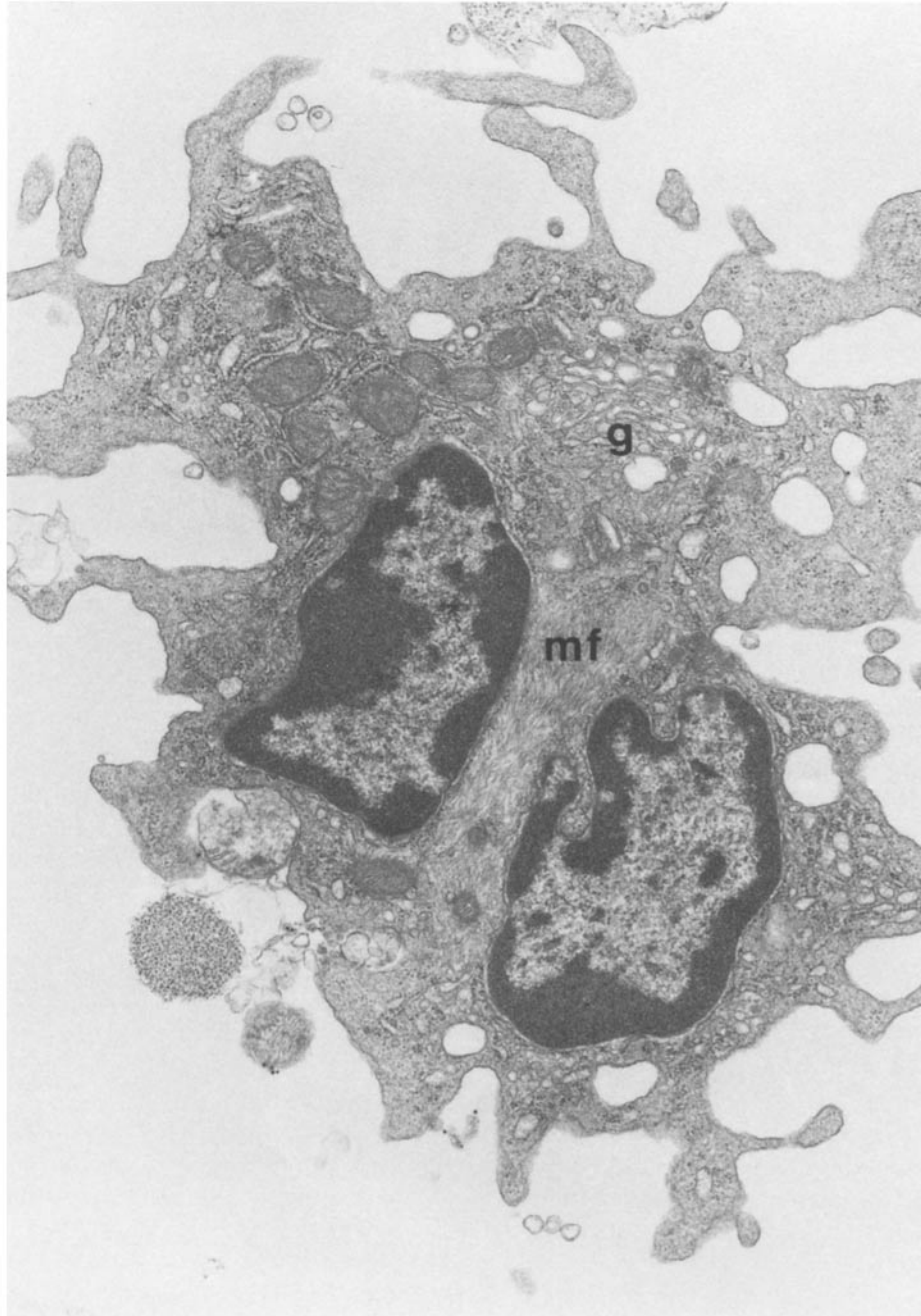
FIGURE 5.   DC from lymph nodes 24 h after skin painting with FITC showing prominent Golgi (g) and microfilaments (mf) (× 17,000).

less fluorescent populations 16 h after skin painting (Fig. 6b). DC expressing high amounts of antigen very effectively induced proliferation whereas the less fluorescent DC were much weaker stimulators, particularly at lower responder
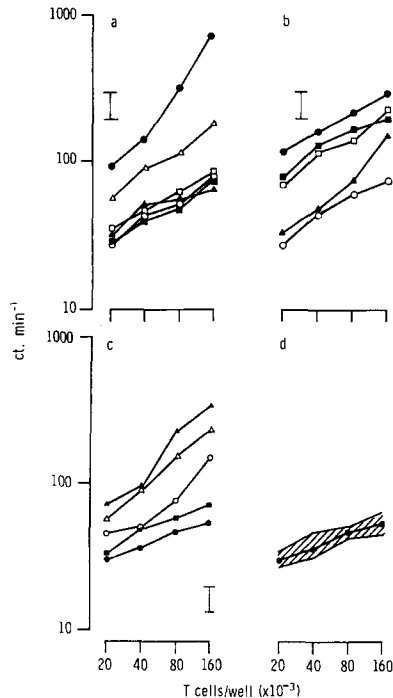
FIGURE 6. Stimulation of normal T cells by irradiated syngeneic DC (1,000) isolated from lymph nodes at various times after skin painting mice with 2 mg FITC. Uptake of [^{3}H]-thymidine in hanging-drop cultures of 2–16 × 10^4 T cells after 3 d. Bar lines show differences significant at $p = 0.01$. (a) Effect of adding DC (1,000) isolated up to 24 h after skin painting. (O) Background; (▲) activity with normal syngeneic DC; (■) DC isolated 30 min after skin painting; (□) DC isolated 2 h after skin painting; (△) DC isolated 8 h after skin painting; (●) DC isolated 24 h after skin painting. (b) Effect of adding DC (1,000) sorted into highly fluorescent and less fluorescent populations 16 h after skin painting. (O) Background; (▲) weakly fluorescent DC; (□) 500 weakly fluorescent DC plus 500 highly fluorescent DC; (■) unsorted DC; (●) highly fluorescent DC. (c) Effect of adding DC isolated from lymph nodes up to 4 d after skin painting mice. (●) Background; (▲) 1 d DC; (△) 2 d DC; (O) 3 d DC; (■) 4 d DC. (d) Effect of adding irradiated T cells isolated from mice after skin painting with FITC. (●) Background. (Shaded region) Effect of adding 1–20 × 10^3 T cells obtained from lymph nodes up to 5 d after skin painting with FITC.

cell concentrations. Reconstituting the sorted DC to the original ratio of 1:1 restored the response to the level of unseparated cells (Fig. 6b).

Maximum proliferation was induced using DC isolated at 24 h after skin painting (Fig. 6c), when all DC expressed a high level of fluorescence. Proliferation was less when DC were obtained between days 2 and 4 when fluorescence was decreasing. No proliferation was detected on subsequent days when fluorescence was close to background levels. There was no significant change in the incorporation of thymidine in normal T cells after the addition of normal syngeneic DC (Fig. 6a) or the addition of T cells obtained up to 4 d after sensitization (Fig. 6d).

Discussion

This work demonstrates a rapid increase in the number of DC in lymph nodes after skin painting with FITC, the preferential location of antigen on the DC, and their potency in initiating immune responses during the first three days after exposure to antigen. At later times the DC were no longer effective and an increase in the number of lymphocytes in the lymph nodes was seen at the time when T cells are able to mediate DTH (reference 4, our unpublished observations).

LC are considered to be precursors of lymphoid DC (19). The route of development of DC may be from skin LC via afferent lymph to node, since DC have been characterized in the lymph where they are called veiled cells (20, 21). It is known that after epicutaneous exposure to antigen, the LC in the epidermis selectively acquire antigens (22). LC with antigen have been reported to be essential for the development of contact sensitivity (23) and have been detected

in the dermal lymphatics 2 h after challenge with contact sensitizers (24), and within the lymph node by 4 h (25). The evolution of LC into afferent lymph veiled cells and then to mature, antigen-presenting DC within the lymph nodes is supported by morphological studies and also by the similarities in function between veiled cells from afferent lymph and DC from lymph nodes (20). Furthermore, Schuler and Steinman (26) have observed the maturation of skin LC in vitro into immunostimulatory cells resembling DC. The appearance of LC in the afferent lymph and in the lymph nodes after skin sensitization is also accompanied by a loss of Ia^+ LC from the skin between 19 h (25, 27) and 3 d after challenge (27).

Our results are compatible with the accepted view that skin LC acquire antigen, migrate via afferent lymph to lymph nodes, and present antigens to T lymphocytes (28–30). The timing of the appearance of DC with high levels of antigen within the lymph nodes coincides with the reported appearance in the lymph nodes of LC (25). The cells with high levels of fluorescence were first seen at 4–8 h, and reached a peak at ~24 h. Using electron microscopy, these fluorescent DC were seen to be more activated than DC from normal mice, were significantly larger, and they expressed large amounts of lysosomes and RER, suggesting that the lysosomal system of DC may not be as poorly developed as previously reported (31). These functionally active cells with high antigen levels were found within the lymph nodes for 3 d, which corresponds to the time period over which depletion of skin LC was reported. Thus, the timing of the appearance of these cells in the lymph node, and their properties in stimulating DTH support the view that the cells are derived from skin LC. Birbeck granules have also been identified in some of our DC preparations. These DC were sensitive to killing by 33D1 antibody plus C. However, this antigen has not been detected on DC matured from skin LC in vitro, which may suggest that 33D1 is expressed on DC after they travel to the lymph node. Alternatively, the sensitivity to 33D1 and the presence of many cells without Birbeck granules could indicate that some DC acquire antigen within the node and are not derived from migratory LC. There was, however, a real increase in the total number of large cells within the viable lymph node cell population at 24 h, and these were shown to be DC. This supports the idea of a migratory pathway for DC at this time, rather than a difference in the efficiency of the isolation procedure.

In addition to the cells with high levels of antigen that stimulated T cells, we also identified cells with low levels of FITC, which did not stimulate responses. These cells were found as early as 30 min after exposure to antigen, the sharpest rise occurring during the first 2 h after skin painting. We do not have evidence as to whether this early peak is due to cells with antigen migrating into the node. These cells had low levels of fluorescence but did not stimulate immune responses. The fluorescence of these DC appeared to be due to the presence of FITC and not to an inherent change in the fluorescence properties of the cells during the development of the immune response, as no fluorescence above the levels in cells from untreated mice was detected during the development of DTH to the nonfluorescent contact sensitizer picryl chloride. The observations that these early lightly fluorescent cells were not functional in initiating immune responses may merely reflect the lower levels of antigen present. However, the definition of these two populations of labeled cells on the basis of the timing of

their appearance and the biphasic distribution of antigen on them suggests that they may be separate populations. As already discussed, it seems likely that the high-intensity cells correspond to the migrating LC. The lightly labeled cells may perhaps be DC, already in transit at the time of skin painting. Contact sensitizers are able to conjugate directly to cell surface proteins and free contact sensitizers can be detected in the lymph as early as 15 min after application (32), which may conjugate directly with circulating DC or preferentially to DC within the lymph nodes. The subsequent appearance of functionally active DC with higher levels of fluorescence and showing signs of increased metabolic activity, would suggest that the simple conjugation of antigen with protein on the cell surface is not sufficient for presentation of the antigen but that some form of antigen processing by DC is necessary.

The mechanisms regulating the distribution of DC after skin painting are unknown. The movement of LC from the skin and their accumulation in the lymph nodes after exposure to antigens is unlikely to depend on an interaction with mature T lymphocytes because the increase in DC observed in the lymph node at 24 h was also seen in nude mice (9). The DC with high levels of antigen usually remained in the lymph nodes for up to 3 d, but in one experiment the level of fluorescence on DC isolated from the nodes remained near maximum for 5 d after skin painting. The factors that regulate the disappearance of DC from the lymph node are also unknown. DC that have interacted with antigen can be targets for NK cells (33), and this may be one mechanism by which the antigen-bearing DC are removed. Exposure to natural infections in the animal colony as well as deliberate immunization may affect the function of DC (30) and may also be influencing the number of DC persisting in the lymph nodes. The persistence of antigen in the lymph nodes in an immunogenic form may also be limited by the development of antihapten antibody (34). We have detected anti-FITC antibody in the serum of mice after skin painting and after injecting DC that have acquired antigen (our unpublished observations). This antibody may modulate the removal of lymph node DC carrying antigen.

A variety of cell types have been reported to transfer sensitization for DTH. These include macrophages (5, 35), T cells (3, 4), LC (36), and DC (8–11). Very small numbers of DC are effective at transferring sensitization for DTH (8–10), and the contribution of some DC remaining in other cell preparations is a possibility. <5% of the separated DC population label as macrophages using the F4/80 mAb (13). In addition, removal of DC from the effective populations with 33D1 removed the activity of the cells, suggesting that macrophages were either not involved or only contributing in a subsidiary role (10).

The time at which DC used for stimulation are isolated from the lymph node after primary skin painting determines the response. Similarly, at early times after exposure to antigen transfer of sensitization to naive animals with lymph node cells appears to involve cells providing the stimulus for the cells mediating the DTH response (37–39). DC cause T cell proliferation in vitro and may therefore provide the stimulus for the development of specific T cell clones in vivo. The specificity of the T cell responses to antigen on DC was shown in earlier studies by the specific cytotoxic activity developing in these cultures (9, 10).

At later times after exposure to antigen (3–5 d), T cells have been shown to

transfer the ability to elicit a response (4, 40), probably by acting as direct mediators of DTH. In this study, the T cells carried no detectable antigen nor did they cause stimulation of syngenic cells in vitro, supporting the idea that they do not provide an antigenic stimulus to another cell type but are probably the effector cells. In addition, a proportion of the 3-d T cells expressed IL-2-R and it is these cells that may be responsible for the DTH reaction (41).

Our studies with B cells provide evidence that antigen on Ia-bearing cells may not be a sufficient signal for sensitization. The period during which B cells acquired transient fluorescence varied and may be influenced by the immuno-logical status of the animal at the time of sensitization. Fluorescence on B cells 18 h after skin painting FITC has been reported (15), but we had not detected this in previous studies of the cells isolated at 24 h (10). Here fluorescence was detected on B cells either 1, 2, or 3 d after skin painting in repeated experiments, and did not last longer than 24 h. This is unlikely to be autofluorescence on activated cells, as B cells obtained from animals skin painted with picryl chloride were not fluorescent. B cells that expressed antigen after skin painting failed to transfer sensitization for DTH (15; our unpublished observations), although class II–positive B cells have been reported to be effective in presenting antigen to sensitized cells (42, 43). A class II–positive macrophage line exposed to FITC in vitro acquired amounts of antigen equivalent to that on DC treated similarly. However, while DC induced high levels of proliferation (10), the macrophage line failed to induce proliferative responses in vitro (Goodacre, J., personal communication). Therefore, efficient antigen presentation by DC must involve specialized properties, which may include a capacity to concentrate antigens, to accumulate in the lymph nodes early after exposure, and to cause clustering and activation of specific T cells (44).

Summary

We have examined the cells involved in the development of contact sensitivity to FITC in CBA mice. After skin painting with antigen, the number of dendritic cells (DC) in the draining lymph nodes increased by 30 min, was maximal at 48 h, and returned to normal by 6 d. Derivation of some DC from Langerhans' cells of the skin was indicated from the presence of Birbeck granules observed in some DC isolated 24 h after skin painting. The DC acquired FITC and by 8 h there were two populations, one highly fluorescent and the other less fluores-cent. The highly fluorescent cells were present between 8 h and 3 d after sensitization, and during this period the DC were potent at initiating primary proliferative responses of normal syngeneic T lymphocytes in vitro. Between days 3 and 5 the numbers of lymphocytes in the draining lymph node increased. During this period purified T lymphocytes did not express detectable levels of antigen, but enriched B cell populations expressed antigen transiently on day 1, 2, or 3 after exposure to antigen. The results showed that, during a 3-d period after exposure to antigen, DC expressed antigen and stimulated T cell prolifer-ation. We speculate that low amounts of FITC binding selectively to veiled cells or lymph node DC in the first hours after exposure to antigen are not immuno-genic but that Langerhans' cells acquire high levels of antigen, enter the nodes, and initiate immune responses.

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