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Contact Sensitivity in the Mouse

XI. MOVEMENT OF T BLASTS IN THE DRAINING LYMPH NODES TO SITES OF INFLAMMATION

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Summary. An earlier paper showed that 4 days after immunization with the contact sensitizing agent 'oxazolone' there is a peak in the percentage of cells in the draining lymph nodes that move to sites of inflammation. This was assessed by dissociating the lymph nodes, labelling them with ^{51}Cr and injecting them into mice whose ears were painted within an hour with an unrelated contact sensitizing agent or with croton oil. The ears were then removed at about 18 hours and their radioactivity used as a measure of cell arrival.

The cells that move to sites of inflammation are not macrophages as they are not removed by filtration through cotton wool. They are θ-positive and control studies show that the activity of the anti-θ serum was indeed due to antibody to the θ-antigen. The cells are large and can be labelled both *in vivo* and *in vitro* with [^{125}I]-iododeoxyuridine which is incorporated in the DNA of cells during the S phase of the cell mitotic cycle. It was concluded that the cells in immunized lymph nodes that move to sites of inflammation are T blasts. The unitary hypothesis is put forward that following immunization for delayed hypersensitivity a particular class of T cells proliferates and gives rise to large pyroninophilic blast cells; and that these cells or their immediate descendants possess the properties, either at the same time or at closely related times, of movement to sites of inflammation and non-specific cytotoxicity as well as the capacity to passively transfer cellular immunity.

INTRODUCTION

The starting point for the present study was the paradox that the spleen and draining lymph nodes are the loci of most of the cell division following immunization with contact sensitizing agents (Asherson and Barnes, 1973), while the peritoneal exudate is the best population for the passive transfer of contact sensitivity (Ptak and Asherson, 1969). Asherson and Allwood (1972) found that following immunization for delayed hypersensitivity, cells appeared in the draining lymph nodes which moved to sites of inflammation. The activity of these cells reached a peak 4 days after immunization with the contact sensitizing agent oxazolone. The question arose whether these particular cells which moved to sites of inflammation belonged to the monocyte macrophage series or were derived from the T lymphocytes which are known to proliferate in immunized lymph nodes.

This paper shows that the cells in immunized lymph nodes that move to sites of inflammation are large, θ-positive (thymus-derived) lymphocytes whose precursors synthesized DNA 2–4 days after immunization. The unitary hypothesis is advanced that non-specific cytotoxicity, movement to sites of inflammation and specific mediation of delayed hypersensitivity are all properties of the same T cell line.

MATERIALS AND METHODS

The general methods follow Asherson and Allwood (1972).

Animals

CBA mice at least 6 weeks old bred at the Clinical Research Centre, The London Hospital Medical College or purchased from Animal Supplies Limited were used. AKR mice were purchased from Jackson Laboratories.

Immunization of donors

Donor mice were immunized with a 3 per cent solution in alcohol of 2-ethoxymethylene-4-phenyloxazolone (oxazolone) (obtained from British Drug Houses) by painting with 0·1 ml onto the skin of the clipped abdomen and one drop or 0·05 ml on each forepaw.

Labelling with ^{51}Cr

The draining inguinal and shoulder girdle lymph nodes were taken 4 days after immunization, dissociated and labelled with $Na^{51}CrO_4$. After two centrifugations the labelled cells were counted and $3–5 \times 10^6$ live cells (assessed with 0·1 per cent trypan blue in 0·2 per cent foetal bovine serum) were injected into each of four to six mice whose ears were painted on both sides within an hour with 2 per cent picryl chloride in olive oil. Eighteen hours later the ears were removed at the point where the cartilage thickens. The limitations of this method are described in Asherson and Allwood (1972).

Labelling with [^{125}I]-iododeoxyuridine

In vitro: [^{125}I]5-iodo-2′-deoxyuridine (IUDR) (1–6 mCi/mg: Radiochemical Centre, Amersham) was added to 2×10^8 cells in 5 ml of 199 medium containing 10 per cent foetal bovine serum. After 2 hours at 37° the cells were spun down and then washed twice.

In vivo: Mice were immunized with oxazolone and lymph nodes taken 4 days later. They were injected with 10 μCi IUDR at various times before killing. The lymph nodes from three mice were removed, dissociated, spun down and washed once before injection into groups of three recipients.

Filtration of lymph node cells

Absorbent cotton wool was prepared by boiling in water and rinsing with alcohol (Hogg and Greaves, 1972). Two hundred milligrams of cotton wool were loosely packed into a 2-ml plastic syringe which already contained medium so as to avoid the formation of air bubbles. The syringe was incubated at 37° for 30 minutes and rinsed with 2 ml of warm medium containing 20 per cent foetal bovine serum. Two millilitres of cells (total 10^8) were added and the syringe incubated for 30 minutes. The cells were then rinsed out

with 2 ml of warm medium containing 20 per cent foetal bovine serum. About 40 per cent of the cells were lost.

Treatment of cells with anti-θ serum

Preparation of anti-θ serum. See Raff (1971). AKR mice were given 10×10^6 live CBA thymus cells intraperitoneally weekly for 7 weeks. A peritoneal exudate was produced by injecting 5×10^6 ascites tumour cells and fluid harvested 5–7 days later, spun at 10,000 ***g*** for 30 minutes, heated at 56° for 30 minutes and stored in aliquots. The ascitic fluid is called anti-θ serum in the text.

Complement: One millilitre of fresh guinea-pig serum was diluted with 2 ml saline and absorbed with 80 mg Ionagar No. 2 (Oxoid) at 4° for 1 hour following Cohen and Schlesinger (1970). It was then absorbed for 1 hour at 4° with a mixture of CBA mouse cells: 75×10^6 thymus cells, 150×10^6 spleen cells, 50×10^6 bone marrow cells and 5×10^9 red cells. The absorption with agar removed nearly all the cytotoxicity of the guinea-pig complement as judged by ^{51}Cr release from labelled normal lymph node cells (unabsorbed complement 38 per cent release; absorbed complement 12 per cent release). The absorption with cells reduced this cytotoxicity to 6–8 per cent which was the same as a buffer control containing 1 per cent foetal bovine serum. The complement was stored in aliquots at −70°.

Validation in vitro *of anti-θ serum.* Normal CBA thymus and lymph node cells were labelled with $Na^{51}CrO_4$, spun down, washed twice and made to a concentration of 50×10^6/ml in Oxoid veronal buffered saline containing calcium and magnesium (Oxoid CFT buffer tablets) with 1 per cent heat inactivated foetal bovine serum. Aliquots of 50 μl of the cell suspension were placed in plastic tubes and 50 μl dilutions of anti-θ serum in the same buffer added. After 30 minutes in the water bath at 37° buffer was added and the cells spun down. One hundred microlitres of absorbed complement (which is a 1/3 dilution) was added and incubated at 37° for 30 minutes. Two millilitres of saline was then added and the cells spun down. A measured quantity of the supernatant was removed and counted. The percentage lysis was given by:

$$\frac{Cr_{exp} - Cr_{con}}{Cr_{100\ per\ cent} - Cr_{con}} \times 100$$

where Cr_{exp} is the release caused by anti-θ serum and complement, Cr_{con} is the release caused by complement alone and $Cr_{100\ per\ cent}$ is the release caused by Triton X–100 (British Drug Houses). Anti-θ serum lysed 91 per cent of thymus cells when used in concentrations between 1/10 and 1/320 and 68 per cent even at a dilution of 1/1280.

Standard conditions for use of anti-θ serum: 2×10^8 cells in 1 ml of 199 were mixed with 1 ml of 1/3 dilution of anti-θ serum and incubated in a water bath for 30 minutes with occasional shaking. The cells were diluted, spun down and resuspended in 2 ml of 1/3 complement, incubated in a water bath for 30 minutes, diluted, spun down, resuspended and counted before labelling with ^{51}Cr. Control cells were similarly handled but foetal bovine serum was used instead of the anti-θ serum and complement. Under these conditions about 55 per cent of the lymph node cells were killed.

Special absorption of anti-θ serum: Serum was absorbed twice with 100×10^6 thymus or nucleated bone marrow cells for 1 hour at 4°. CBA liver was homogenized at 1/15 w/v in iced phosphate buffered saline, centrifuged at 2500 ***g*** for 15 minutes and washed three times. CBA and AKR brain was prepared similarly but washed five times. Serum was

absorbed twice with 1/5 of its volume of packed liver. Serum diluted to 1/3 was absorbed once with 1/5 of its volume of packed brain. See Raff and Greaves (1972).

Separation of cells by size: The method of Miller and Phillip (1969) was used. Cells were loaded into a 6-cm radius settling chamber obtained from GK Scientific, Shirley, Warwickshire. The following fluids were run in under gravity: (a) an overlay of 20 ml of phosphate buffered saline (Dulbecco's PBS); (b) $2{\cdot}5 \times 10^8$ immunized lymph node cells in 0·2 per cent bovine serum albumin (Armour, Fraction V) in 20 ml PBS; (c) 50 ml of 0·5 per cent bovine serum albumin, 4 per cent heat inactivated foetal bovine serum and 40 per cent RPMI 1640 (Biocult, Glasgow) in PBS; (d) 50 ml of 1 per cent bovine serum albumin in medium (c); (e) 100 ml of 1·5 per cent bovine serum albumin in medium (c); (f) 150 ml of 2·0 per cent bovine serum albumin in medium (c).

The cells were allowed to sediment for 2·5 hours, taken off in 10 ml fractions, recovered by centrifugation, pooled into four groups and washed once before labelling with ^{51}Cr.

RESULTS

CELLS IN IMMUNIZED LYMPH NODES THAT MOVE TO SITES OF INFLAMMATION ARE LYMPHOCYTES AND NOT MACROPHAGES

Lymph node cells were obtained 4 days after skin painting with the contact sensitizing agent oxazolone. They were dissociated and labelled with ^{51}Cr and then injected intravenously into syngeneic recipients. Sites of inflammation were then produced by painting

TABLE 1

LYMPH NODE CELLS 4 DAYS AFTER IMMUNIZATION WITH OXAZOLONE. THE EFFECT OF FILTRATION ON THEIR MOVEMENT TO SITES OF INFLAMMATION

Treatment	Percentage arrival at sites of inflammation
Nil	1·0 ±0·13
Filtered	0·88±0·15

The figures show the mean net percentage ± SD of the injected radioactivity (^{51}Cr) in the two ears at 18 hours. Filtration did not cause a significant reduction in arrival.

the ears with picryl chloride. The ears were removed 18 hours later and their radioactivity expressed as a percentage of the injected radioactivity. This is a measure of the net percentage cell arrival providing ^{51}Cr labels different cells equally and is not eluted. The percentage of cells that move to sites of inflammation reaches a peak four days after immunization (Asherson and Allwood, 1972). For this reason lymph nodes were used four days after immunization with oxazolone in the following experiment.

Table 1 shows that 1 per cent of immunized lymph node cells arrived at inflammed ears. This arrival was not significantly reduced by filtration through cotton wool. It was concluded that the cells in the immunized lymph nodes that moved to sites of inflammation were lymphocytes and not macrophages or other cells that adhere readily to cotton wool.

THETA-POSITIVITY OF IMMUNIZED LYMPH NODE CELLS THAT MOVE TO SITES OF INFLAMMATION

Table 2 shows that treatment of immunized lymph node cells with anti-θ serum and complement reduced the arrival at inflammed ears from 0·85 to 0·12 per cent, a reduction of 86 per cent. It also shows that the activity of the anti-θ serum was removed by absorption with CBA thymus or brain, but not by absorption with CBA bone marrow or AKR thymus or brain. In a separate, unreported experiment, CBA liver also failed to absorb activity. It was concluded that the lymphocytes that moved to sites of inflammation were θ-positive.

TABLE 2

LYMPH NODE CELLS 4 DAYS AFTER IMMUNIZATION WITH OXAZOLONE. THE EFFECT OF TREATMENT WITH ANTI-θ SERUM ON MOVEMENT TO SITES OF INFLAMMATION

Sera added during incubation		Percentage arrival at sites of inflammation	
		Exp. 1	Exp. 2
Nil	(negative control)	0·85 ± 0·03	0·73 ± 0·10
Complement only	(negative control)	0·83 ± 0·11	0·63 ± 0·001
Anti-θ	(experiment)	0·12 ± 0·02	0·21 ± 0·05
Anti-θ absorbed with CBA thymus	(specificity control)	0·71 ± 0·11	
Anti-θ absorbed with CBA bone marrow	(specificity control)	0·20 ± 0·15	
Anti-θ absorbed with AKR thymus	(specificity control)	0·11 ± 0·04	
Anti-θ absorbed with CBA brain	(specificity control)	0·90 ± 0·093	
Anti-θ absorbed with AKR brain	(specific control)	0·28 ± 0·013	

All incubations with anti-θ serum were followed by incubation with complement. The data show that anti-θ serum reduced cell arrival and that this reduction was prevented by absorption with CBA thymus or brain but not by absorption with CBA bone marrow or AKR thymus or brain.

IMMUNIZED LYMPH NODE CELLS THAT MOVE TO SITES OF INFLAMMATION ARE LARGE CELLS

Immunized lymph node cells were separated by velocity sedimentation and then labelled with ^{51}Cr. Table 3 shows that the unfractionated cells gave an arrival at sites of inflammation of 0·55 per cent. The arrival of fraction 1 (largest cells) was 0·79 per cent and of fraction 4 (smallest cells) was 0·19 per cent. These results suggest that individual large cells have a greater movement to sites of inflammation than small cells. An alternative interpretation is that fraction 1 (largest cells) has a relatively higher percentage of the cells that move to sites of inflammation than fraction 4 (smallest cells).

IMMUNIZED LYMPH NODE CELLS THAT MOVE TO SITES OF INFLAMMATION ARE DERIVED FROM CELLS THAT RECENTLY SYNTHESIZED DNA

In vitro *labelling with IUDR*: Immunized lymph node cells were incubated with [^{125}I]-IUDR for a 2-hour period and injected into recipient mice. Table 4 shows that their

TABLE 3

LYMPH NODE CELLS 4 DAYS AFTER IMMUNIZATION WITH OXAZOLONE. THE ABILITY OF FRACTIONS PREPARED BY VELOCITY SEDIMENTATION TO MOVE TO SITES OF INFLAMMATION

Cell population	Percentage arrival at sites of inflammation
Initial cells	0·55 ± 0·09
Fraction 1 (largest cell size)	0·79 ± 0·05
Fraction 2	0·27 ± 0·04
Fraction 3	0·28 ± 0·04
Fraction 4	0·19 ± 0·02

The initial population was kept at 4° during the fractionation procedure. These data are quoted in Asherson and Ferluga (1973).

TABLE 4

LYMPH NODE CELLS 4 DAYS AFTER IMMUNIZATION. MOVEMENT OF CELLS LABELLED *in vitro* WITH [^{125}I]-IODODEOXYURIDINE AND ^{51}Cr TO SITES OF INFLAMMATION

Cell label	Percentage arrival at sites of inflammation caused by		
	Nil (unchallenged)	Picryl chloride	Croton oil
[^{125}I]-iododeoxyuridine	0·12 ± 0·05	1·9 ± 0·25	2·8 ± 0·64
^{51}Cr	0·02 ± 0·005	0·48 ± 0·09	0·68 ± 0·14

Separate aliquots of cells were labelled with IUDR or ^{51}Cr and mixed before injection. Three per cent croton oil in dibutylpthhalate was used.

TABLE 5

LYMPH NODE CELLS 4 DAYS AFTER IMMUNIZATION WITH OXAZOLONE. MOVEMENT OF CELLS LABELLED WITH ^{125}I-IODODEOXYURIDINE *in vivo* TO SITES OF INFLAMMATION AT VARIOUS TIMES AFTER IMMUNIZATION

Time of labelling after immunization (hours)	Time of labelling before harvesting (hours)	Percentage arrival at sites of inflammation
48	48	1·82 ± 0·22
72	24	1·28 ± 0·96
78	18	1·22 ± 0·17
88	8	2·19 ± 0·30
92	4	2·06 ± 0·48

arrival at sites of inflammation was 0·8 per cent. In contrast, cells labelled in parallel with ^{51}Cr instead of IUDR gave an arrival of 0·5 per cent. It was concluded that cells that had recently synthesized DNA showed a greater movement to sites of inflammation than the average lymph node cell.

In vivo *labelling with IUDR*: Groups of mice were immunized with oxazolone and their lymph nodes taken at 4 days. Each group received a single injection of IUDR at times ranging from 48 to 4 hours before taking their lymph node cells for injection into recipient mice. Table 5 shows that 1·2–2·2 per cent of the labelled lymph node cells moved to sites of inflammation regardless of the time of administration of the IUDR. It was concluded that at least some of the cells that move to sites of inflammation were synthesizing DNA between 48 and 88 hours after immunization; and that cells synthesizing DNA 4–48 hours before injection move to sites of inflammation. It is likely that most of these DNA synthesizing cells go on to cell division.

DISCUSSION

Asherson and Allwood (1972) studied the draining lymph nodes of mice immunized with oxazolone and other agents which cause delayed hypersensitivity. These nodes contain cells which move to sites of inflammation (ears painted with picryl chloride) when injected into recipient mice. The present results show that the cells found 4 days after immunization with oxazolone are large, thymus-derived cells which arise from DNA synthesizing precursors.

Filtration through cotton wool showed that the cells that move to sites of inflammation were not macrophages or other cells which adhere readily to cotton wool. Their sensitivity to anti-θ serum and complement was *prima facie* evidence that they were T lymphocytes. Specificity controls were, however, performed to exclude other possibilities. See Raff (1971), Raff and Greaves (1972), and Dennert and Lennox (1972).

The cells that moved to sites of inflammation were large cells. This suggested that they were in the S phase of the mitotic cycle. This was confirmed by showing that cells which were synthesizing DNA, as judged by the uptake of [^{125}I]iododeoxyuridine (IUDR), moved to sites of inflammation. The fact that cells were labelled whether the IUDR was given 48, 24, 18, 8 or 4 hours before injection is compatible with their origin from a rapidly dividing line of cells within the lymph nodes.

The cells that move to sites of inflammation are T cells and the finding that they are large cells that recently synthesized DNA suggests that they are thymus-derived blast cells. The large pyroninophilic cells found in lymph nodes 4 days after immunization with oxazolone are also blast cells derived from T cells. This suggests that cells that move to sites of inflammation may be identical to the large pyroninophilic cells or their immediate descendants. This hypothesis is best considered against the background of the morphological and functional changes which occur in lymph nodes following exposure to antigens that cause delayed hypersensitivity.

Following immunization with contact sensitizing agents T cells proliferate and become large pyroninophilic blast cells. DNA synthesis reaches a peak at day 3 (Pritchard and Micklem, 1972; Asherson and Barnes, 1973) and the number of large pyroninophilic cells in the paracortical area reaches a peak 1 day later (Davies, Carter, Leuchars and Wallis, 1969; Turk, 1967).

Some of these cells leave the lymph nodes and appear in the thoracic duct. There is a

sharp peak at 4½ days in the percentage of large pyroninophilic cells in the thoracic duct lymph in rats immunized with the contact sensitizing agent dinitrochlorobenzene (Delorme, Hodgett, Hall and Alexander, 1969). Sprent and Miller (1972a) made similar observations in irradiated F1 mice given parental thymus cells. In this system the injected cells respond to the transplantation antigen in the F1 hybrid and become large, rapidly dividing, specifically cytotoxic cells which appear in the thoracic duct in large numbers four days after injection. By day 6 most of the large pyroninophilic cells have left the lymph node or reverted to small lymphocytes. This reversion has been studied in the guinea-pig with contact sensitivity (Turk, 1967) and in the mouse with graft versus host reactions (Gowans, 1965; Sprent and Miller, 1972b).

The lymphocytes that move to sites of inflammation share many of the properties of large pyroninophilic cells found in the paracortical area of immunized lymph nodes. Both appear in lymph nodes responding to antigens that cause delayed or contact sensitivity and both are abolished by the induction of unresponsiveness which prevents the development of delayed hypersensitivity. The evidence for this is that the cells that move to sites of inflammation do not appear in the lymph nodes of mice rendered unresponsive by picryl sulphonic (trinitrobenzene sulphonic) acid when they are subsequently painted with picryl chloride (Asherson and Allwood, 1972). Similarly large pyroninophilic cells do not appear in the lymph nodes of guinea-pigs rendered unresponsive by dinitrobenzene-sulphonic acid when they are subsequently painted with dinitrofluorobenzene, the dinitrofluoro analogue of picryl chloride (trinitrochlorobenzene) (Turk, 1967).

Both large pyroninophilic cells and cells that move to sites of inflammation are large, thymus derived, blasts which arise from a cell line which synthesizes DNA, and presumably divides, 2–4 days after immunization. The peak number of large pyroninophilic cells and the greatest percentage movement to sites of inflammation both occur 4 days after immunization. Another property which reaches a maximum on day 4 is non-specific cytotoxicity. This is due to large theta-positive cells which on indirect evidence are derived from a line of cells which synthesize DNA (Asherson, Ferluga and Janossy, 1973).

Our current views are summarized by the unitary hypothesis which states that following immunization for delayed hypersensitivity a particular class of T cells proliferates and gives rise to large pyroninophilic blast cells. These cells or their immediate descendants possess the properties, either at the same time or closely related times, of movement to sites of inflammation and of non-specific cytotoxicity. The unitary hypothesis can be contrasted with the pluralistic hypothesis, which in its extreme form states, that each property of T lymphocytes in immunized lymph nodes, such as movement to sites of inflammation, non-specific cytotoxicity, specific cytotoxicity (Alexander, Bensted, Delorme, Hall and Hodgett, 1969) and passive transfer of cell-mediated immunity is due to a different line of T cells derived from a distinct subclass which was present before immunization.

The unitary hypothesis is in keeping with the data and interpretations of several authors. Koster and McGregor (1971) suggested that the large and medium size blast cells which appear in the thoracic duct one week after immunization with bacteria were the same cells as those which moved to sites of inflammation (Koster, McGregor and Mackaness, 1971) and which passively transferred resistance to bacteria (McGregor, Koster and Mackaness, 1971). Werdelin, Wick and McCluskey (1971) made similar observations by labelling lymph node cells *in vivo*. Sprent and Miller (1972c) showed that blasts derived from T cells, in the thoracic duct, were specifically cytotoxic and suggested that these cells were analogous to those studied by Koster and McGregor (1971). Rosenstreich,

Blake and Rosenthal (1971) provided indirect evidence for the concept that the cells, that mediate delayed hypersensitivity and its *in vitro* correlates, move to sites of inflammation.

The blasts derived from T cells which are found in immunized lymph nodes are only one of the types of mononuclear cells that move to sites of inflammation. Spector and Willoughby (1968) and Volkmann (1966) have shown that cells of the monocyte-macrophage series whose precursors lie in bone marrow also move to sites of inflammation. It is an interesting question what classes of lymphocytes have the ability to move to sites of inflammation other than blast cells derived from T cells.

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