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The Effect of Localized Injection of Adjuvant Material on the Draining Lymph Node

II. CIRCULATING LYMPHOCYTES

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Summary. The stimulation of a draining lymph node by the injection of a substance possessing adjuvanticity leads to a marked increase in the influx of lymphocytes into the node. The influx of cells has been followed in experimental mice injected intravenously with ^{51}Cr-labelled normal mesenteric lymph node cells. The increase in size of the draining node is not due to an increased blood volume.

INTRODUCTION

In an earlier paper (Taub, Krantz and Dresser, 1970) we presented the histological evidence which led us to the conclusion that the enlargement of a popliteal lymph node draining a site where a substance with extrinsic adjuvanticity had been injected was due to the influx of circulating lymphocytes. A marked paracortical hypercellularity was observed which preceded blast cell transformation and cell division by several hours. Moreover, the observed lymph node enlargement and hypercellularity of the paracortical region was accompanied by a marked dilatation of post-capillary venules, the route of cell migration suggested by Gowans and Knight (1963). It seemed desirable that these conclusions based on histological findings by both us and others, in particular Eidinger (1968) who has studied the local draining node after the injection of xenogeneic erythrocytes, should be confirmed by experimental procedures. We, therefore, made a brief study of the short-term distribution of normal ^{51}Cr-labelled lymphocytes and thymocytes in mice injected locally with adjuvant materials.

Despite the possibility of a relatively high rate of elution of ^{51}Cr from labelled lymphocytes in certain situations (Maupin and Loverdo, 1959), it has been shown that this label can be used to follow the quantitative distribution of lymphocytes *in vivo* (Bainbridge, Brent and Gowland, 1966; Ronai, 1969). Because of the non-specific elution of label, the experimental design should be symmetrical to allow comparison with valid controls and the time between the injection of labelled cells and the measurement of ^{51}Cr in target organs should not be more than 48 hours and preferably 24 hours or less. Lance and Taub (1969) have shown that only a relatively small proportion of a population of injected labelled lymphocytes can recirculate in normal recipients and localize in the lymphoid organs of the host, probably entering the parenchyma of lymph nodes by traversing the post-capillary venules (Gowans and Knight, 1964). The size of this recirculating subpopulation is different in different lymphoid organs, for instance thymi and mesenteric lymph nodes; a point made by both Goldschneider and McGregor (1968) and Lance and Taub (1969).

MATERIALS AND METHODS

Male CBA mice, 3–6 months old, were used in these experiments. Test substances were injected into the right footpad as described in the previous paper (Taub *et al.*, 1970), where the type and degree of adjuvanticity of these substances is listed and discussed. Sheep and CBA mouse red blood cells, which were not used in the earlier experiments, were prepared from whole blood in Alsever's solution by washing twice with citrate saline (0·15 M) and then twice with Gey's solution; each wash consisted of centrifugation at 1750 ***g*** for 5 minutes followed by removal of the supernatant and the buffy coat.

Lymph node or thymus cell suspensions were labelled with ^{51}Cr by a method similar to that of Bainbridge and Gowland (1966). The cells were suspended in Eagle's minimal essential medium (MEM) buffered at pH 7·3–7·4 with tris (0·05 M) and containing 20 per cent foetal calf serum. The cells were incubated with 10–100 μCi/100 $\times 10^6$ cells at 37° for 30 minutes. The temperature of the cells was then reduced to 0–4° and they were then washed twice by centrifugation at 600 ***g*** and decantation of the supernatant before 0·5–2 $\times 10^7$ cells were injected intravenously into syngeneic recipients. Twenty-four hours later the recipient mice were killed and their spleens, peripheral (brachial, axillary, inguinal and popliteal) lymph nodes, mesenteric lymph nodes, liver and in some cases the thymus were removed and individual assessment was made of the amount of ^{51}Cr contained by these organs. The activity in each organ of a group of mice was expressed as the geometric mean percentage of the total activity injected 24 hours previously. Estimates of the amount of activity in blood were made from measured volumes of whole blood obtained by cardiac puncture. CBA erythrocytes were labelled with ^{51}Cr by a similar method using a concentration of 5 $\times 10^8$ RBC/ml and using 5 μCi $^{51}Cr/10^8$ cells.

RESULTS

Groups of four mice were injected with 2·5 $\times 10^8$ pertussis organisms in the right footpad, 6, 3, 2 and 1 day before, 1 hour before and 1 hour after the intravenous injection of 1·5 $\times 10^7$ ^{51}Cr-labelled mesenteric lymph node cells. Twenty-four hours later, the right (draining, ipsilateral) and the left (control, contralateral) popliteal lymph nodes were removed. Each node was weighed on a 0–10 mg torsion balance before the amount of ^{51}Cr radioactivity was measured in a 'Packard Autogamma' scintillation spectrometer. Activity was expressed as a percentage of the total injected 24 hours previously. The geometric mean (log x) weight and percentage activity are plotted together in Fig. 1. A similar experiment, on a more limited scale, comparing labelled mesenteric node cells and thymus cells is summarized in Table 1. There is a qualitative similarity between the weight and activity curves in both sets of nodes.

Table 2 shows that thymocytes distribute themselves in normal syngeneic mice rather differently from mesenteric lymph node cells. Thymocytes show a lesser tendency to localize in lymph nodes than do lymphocytes and a greater tendency to localize in the spleen, confirming the earlier observations of Lance and Taub (1969). The overall distribution of thymocytes and lymphocytes does not alter when the mice have been injected 6 days previously in the right footpad with pertussis, although this time interval may be too great on the basis of the results illustrated in Fig. 1. The increased localization of labelled cells in the draining popliteal nodes is apparent with both thymocytes and lymphocytes. Furthermore, it appears that a local injection of a mixture of pertussis organisms and supernatant decreases the localization of thymocytes in the mesenteric lymph node.

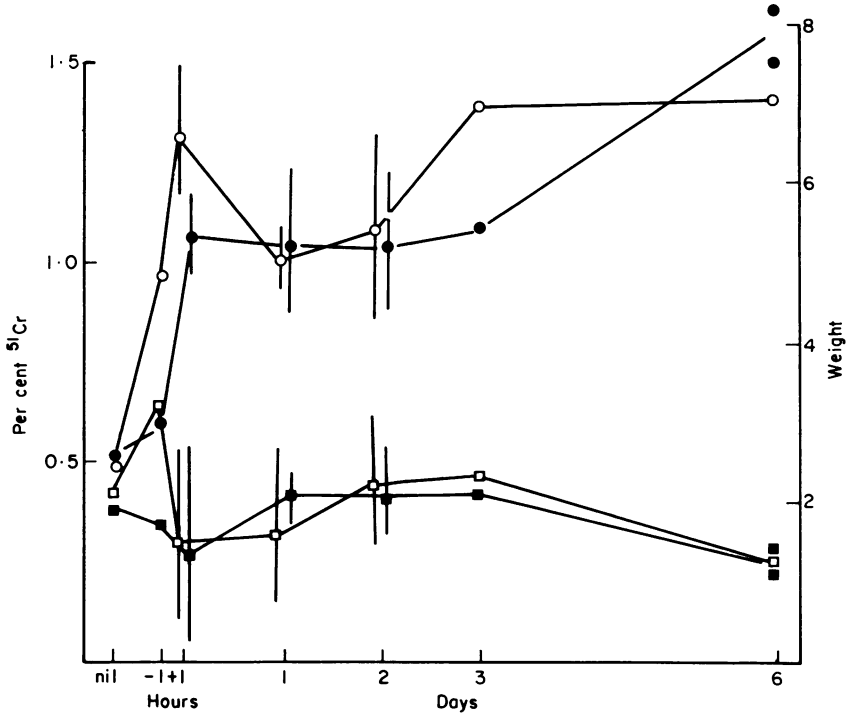

FIG. 1. The change in popliteal lymph node weight and in the localization of labelled normal mesenteric lymph node cells in ipsilateral (right-draining, circles) and contralateral (left-control, squares) popliteal nodes of mice injected with pertussis in the right footpad. The time scale is the time between the injection of pertussis and the intravenous injection of labelled mesenteric lymph node cells. Open symbols are the geometric mean weight (mg) of the nodes, and closed symbols are the geometric mean activity expressed as a percentage of that injected 24 hours previously. Vertical bars indicate standard deviation.

TABLE 1

THE DISTRIBUTION OF ^{51}Cr-LABELLED MESENTERIC LYMPH NODE CELLS AND THYMUS CELLS IN THE POPLITEAL LYMPH NODES OF MICE INJECTED WITH PERTUSSIS IN THE RIGHT FOOTPAD

	Popliteal lymph node	Time between injection of $2·5 \times 10^8$ pertussis into the right footpad and the i.v. injection of labelled cells*		
		2 hours	1 day	3 days
Weights (mg) A+B	Control	1·1 (2·1–0·5) [8]	1·3 (3·4–0·6) [8]	1·4 (3·2–0·4) [8]
	Draining	3·3 (6·5–1·5) [7]	4·3 (14·1–0·8) [8]	7·8 (13·9–4·2) [8]
A Counts/min	Control	169 (420–69) [4]	299 (540–166) [4]	279 (827–94) [4]
	Draining	490 (747–320) [3]	1006 ( ) [1]	1280 (1836–894) [4]
B Counts/min	Control	13 (138–2) [4]	4 (95–<1) [4]	39 (158–10) [4]
	Draining	46 (181–12) [4]	147 (512–43) [4]	176 (262–119) [4]

Group A Injected with 25×10^6 mesenteric lymph node cells, i.v.; total count 138,000/min.
Group B Injected with 25×10^6 thymus cells, i.v.; total count 170,000/min.

*Control (left) and draining (right) popliteal nodes removed 22–24 hours later. Geometric mean weight and count tabulated with 95 per cent confidence levels in parentheses and the number of individuals in each group in brackets. Background is 40 counts/min.

TABLE 2

ORGAN WEIGHT AND DISTRIBUTION OF ^{51}Cr-LABELLED NORMAL SYNGENEIC THYMOCYTES (T) OR MESENTERIC LYMPH NODE CELLS (L) AFTER INTRAVENOUS INJECTION OF 10^7 CELLS INTO MICE INJECTED IN THE RIGHT FOOTPAD WITH VARIOUS SUBSTANCES 3 OR 6 DAYS PREVIOUSLY (ACTIVITY EXPRESSED AS A PERCENTAGE OF THE TOTAL ACTIVITY INJECTED 24 HOURS BEFORE)

Substance injected into right footpad [day -6 (except for Vitamin A)]	Cells injected (day 0)	*Organ weight (mg) popliteal lymph nodes		*Activity (per cent) (day+1)			
						Popliteal lymph nodes	
		Right (draining)	Left (control)	Peripheral nodes	Mesenteric nodes	Right (draining)	Left (control)
Nil (control)	T	ND	N/D	0·4 (0·69–0·23)	0·7 (0·93–0·54)	0·025 (0·22–0·004)	0·018 (0·08–0·004)
$2{\cdot}5 \times 10^8$ pertussis	T	N/D	N/D	0·8 (1·20–0·52)	0·1 (0·13–0·08)	0·100 (0·23–0·04)	0·049 (0·11–0·02)
Nil (control)	L	2·5 (5·8–1·1)	2·7 (4·5–1·6)	5·2 (7·9–3·5)	8·7 (15·1–5·0)	0·23 (1·7–0·03)	0·34 (1·1–0·11)
$2{\cdot}5 \times 10^8$ pertussis	L	N/D	N/D	3·7 (5·4–2·6)	7·0 (10·7–4·7)	1·66 (2·8–1·0)	0·22 (2·2–0·02)
5×10^6 sheep RBC	L	6·3 (15·8–2·5)	1·4 (1·8–1·1)	4·9 (7·4–3·2)	7·6 (10·7–5·5)	1·21 (2·3–0·63)	0·38 (1·05–0·14)
5×10^6 CBA mouse RBC	L	2·4 (3·6–1·6)	1·8 (3·3–1·0)	5·0 (6·9–3·6)	7·7 (10·2–5·9)	0·56 (0·62–0·51)	0·39 (0·58–0·28)
Alum particles without antigen	L	4·5 (5·9–3·4)	2·2 (4·0–1·2)	4·5 (6·2–3·2)	7·4 (10·2–5·4)	1·05 (1·2–0·91)	0·39 (0·78–0·20)
0·5 mg Vitamin A alcohol in 0·025 ml liquid paraffin day −3	L	2·4 (5·0–1·1)	1·8 (2·6–1·2)	4·3 (5·9–3·1)	6·2 (13·5–2·8)	0·84 (1·6–0·44)	0·26 (1·9–0·04)

N/D = not done. Numbers in parentheses are 95 per cent confidence limits.

* Geometric (log x) means transformed back to arithmetic values; spleen weights and the percentage activity in the spleen and liver were unaffected by the experimental procedures used here, with the exception that the per cent localization of mesenteric lymphocytes in the liver of mice treated with Vitamin A was 12·9 (13·5–12·3) compared with 22·8 (30·2–17·4) in controls.

The effects of injected alum (without antigen), xenogeneic and syngeneic red blood cells and vitamin A are also included in Table 2. It is clear that although the injection of syngeneic red blood cells led to a slight enlargement and a slight increase in percentage activity in the draining node after the intravenous injection of labelled lymphocytes, the xenogeneic (sheep) red blood cells had a much greater effect. Alum and Vitamin A alcohol both stimulated an increase in the weight of the draining node, which was associated with a closely corresponding increase in the percentage activity (^{51}Cr).

In another experiment sixteen CBA mice were injected in the right footpad with $2{\cdot}5 \times 10^8$ pertussis organisms. Six days later these mice were injected intravenously with 30×10^6 heavily labelled mesenteric lymphocytes ($100\,\mu Ci/10^8$ cells). After 24 hours, the right and left nodes were removed and pooled separately. After homogenization of the nodes in Gey's solution the cells ($1{\cdot}5 \times 10^7$) were injected intravenously into two groups of four untreated CBA mice. The following day the peripheral nodes, mesenteric nodes, spleen, liver and popliteal nodes were removed and the activity measured as before. It is clear that at least the great majority of the labelled cells from the draining nodes behave similarly to those from the contralateral (control) nodes, and to normal lymphocytes. This result is shown in Table 3. An attempt was made to see if cells from draining node would tend to 'home' to draining rather than to control nodes or to any other lymphoid organ. Table 4

TABLE 3

THE FINAL DISTRIBUTION OF LABELLED MESENTERIC LYMPH NODE CELLS WHICH HAVING LOCALIZED IN DRAINING AND CONTROL POPLITEAL LYMPH NODES WERE RE-TRANSFERRED TO NORMAL SYNGENEIC RECIPIENTS

Organs of second recipient	Percentage of counts injected			
	Control*		Draining*	
Peripheral nodes	11·0	(14·4–7·6)	7·5	(9·5–6·0)
Mesenteric nodes	4·1	(6·4–2·6)	6·1	(8·9–4·1)
Spleen	29·0	(31·5–26·5)	24·9	(31·3–19·7)
Liver	12·5	(22·4–8·3)	9·3	(14·7–5·7)
Thymus	0·09	(0·11–0·017)	0·006	(0·026–0·0009)

Geometric means with 95 per cent confidence limits in parentheses.

* Origin of labelled mesenteric lymph node cells localizing in popliteal lymph nodes 21 hours after transfer.

summarizes such an experiment which is basically similar to those summarized in Tables 2 and 3 with the important difference that the final recipients had, 3 days before, been injected with pertussis in the right footpad. Unfortunately, due to the very low recovered counts in the control nodes of mice injected with cells accumulated in control (left) nodes of first stage recipients, the 95 per cent confidence limits are extremely wide. Within the gross limits set by this result there are no differences between the two groups. The slightly (not significant) lower level of counts in lymphoid organs and higher level in the livers of the mice receiving cells accumulating in control (left) popliteal nodes may well be due to a relatively greater amount of mechanical damage received during preparation of the cell suspensions, due to the very much smaller size of the control (left) nodes.

Table 5 shows the distribution of labelled syngeneic RBC 24 hours after intravenous injection in mice injected 5 days previously with $2{\cdot}5 \times 10^8$ pertussis organisms in the right footpad. It can be seen that most of the labelled cells are in the blood and also that the calculated absolute blood volume of draining nodes is slightly less than that of the controls;

TABLE 4

THE DISTRIBUTION IN SYNGENEIC RECIPIENTS OF ^{51}Cr-LABELLED MESENTERIC LYMPH NODE CELLS WHICH HAD BEEN RECOVERED AFTER 24 HOURS FROM THE POPLITEAL LYMPH NODES OF MICE INJECTED IN THE RIGHT FOOT PAD WITH PERTUSSIS AND RE-TRANSFERRED TO MICE WHICH ALSO HAD BEEN INJECTED IN THE RIGHT FOOTPAD WITH PERTUSSIS

Source of popliteal lymph node cells for second transfer	Popliteal lymph nodes				Other lymphoid organs (percentage activity)			
	Percentage activity		Weight (mg)					
	Control	Draining	Control	Draining	Peripheral nodes	Mesenteric nodes	Spleen	Liver
Control (left)	0·28 (1·37–0·06)	0·40 (1·81–0·09)	1·46 (7·30–0·29)	6·66 (41·5–1·07)	2·94 (14·18–0·61)	2·69 (14·21–0·51)	23·7 (13·8–4·9)	30·7 (140·6–6·69)
Draining (right)	0·35 (0·90–0·14)	0·57 (1·39–0·23)	1·78 (5·44–0·58)	6·82 (22·8–2·04)	5·17 (14·0–1·91)	3·82 (9·88–1·47)	26·5 (65·0–10·8)	23·0 (57·5–9·2)

The distribution of label shown in the table was made 24 hours after the second transfer and 4 days after the injection of pertussis into the right footpads of these mice. Geometric means with 95 per cent confidence limits in parentheses.

TABLE 5

THE DISTRIBUTION OF LABELLED CBA-RBC IN THE ORGANS OF MICE INJECTED 5 DAYS BEFORE TRANSFER WITH $2{\cdot}5 \times 10^8$ PERTUSSIS IN THE RIGHT FOOTPAD

Organs	Percentage of counts injected		Mean blood volume (μl)		Blood volume as percentage of node weight	
Peripheral nodes*	0·029	(0·052–0·016)	–		–	
Mesenteric nodes	0·036	(0·051–0·025)	–		–	
Spleen	2·3	(5·74–0·94)	–		–	
Liver	6·3	(11·2–3·4)	–		–	
Blood (per ml)	60·0	(97·7–37·2)	–		–	
Left popliteal node (control)		–	0·075	(0·084–0·047)	2·83	(5·52–1·40)
Right popliteal node (draining)		–	0·029	(0·049–0·017)	0·22	(0·50–0·098)

Re-transformed geometric means with 95 per cent confidence limits in parentheses.

* Excluding the popliteal nodes.

however, it is clear that when the greatly increased size of the draining node is taken into account, there is a striking reduction in the relative blood volume of the draining node. This makes it impossible that the increased size of the draining node and the increased content of lymphocytes is due to an increased vascularity.

DISCUSSION

Our study shows that there is an increased localization of labelled mesenteric lymph node cells (Fig. 1) or thymocytes (Table 1) in popliteal nodes draining a site of adjuvant injection, thus strengthening the conclusion that the initial increase in node size is due to the influx of cells. The amount of the observed increase in size and cellularity of the draining node seems to be compatible with the rate of turnover of circulating lymphocytes in mice calculated by Gesner and Gowans (1962). They showed that about 170×10^6 thoracic duct cells enter the blood in 24 hours. Fig. 1 shows that 1 per cent of mesenteric lymph node cells injected intravenously localize in a draining node in 24 hours. The absolute increase in the number of cells in a draining node 1 day after the injection of pertussis into the footpad is $2–3 \times 10^6$ cells. We conclude from the experiments with labelled syngeneic erythrocytes (Table 5) that the increased size of a draining node is not due to an increase in its blood volume. Observed differences between lymphocytes and thymocytes (Table 2; Lance and Taub, 1969) suggest that the cells migrating to the draining lymph nodes belong to the pool of recirculating lymphocytes.

In the experiments where normal labelled cells localizing in a popliteal node are re-transferred to normal syngeneic recipients, it can be seen (Table 3) that most of those cells which localize in control and draining nodes retain their ability to circulate in a normal manner. It, therefore, seems possible to conclude that residence in the parenchyma of a lymph node does not immediately destroy a cell's ability to recirculate; confirming the previous conclusions of Lance and Taub (1969). The experiments summarized in Table 4 indicate that, at a gross general level, cells from a draining node do not 'home' specifically to a draining node. However, it is still possible that such homing may take place amongst the relatively few cells which may be involved in a specific response: < 1 per cent of the cells in a spleen respond by making antibody to sheep RBC in a hyperimmune response to that antigen (Wortis, Taylor and Dresser, 1966).

The elucidation of the mechanism of cellular influx into a draining node must await further experimentation. The observed enlargement can be due to one of two possible

mechanisms: (1) a change in the physical structure of the node which results in an increase in the efficiency of the mechanical trapping of circulating lymphocytes and also perhaps changes which make space available for circulating cells to settle down; and (2) the secretion of a chemotactic agent which actively stimulates the cells to migrate to and settle down in a draining node. The recirculation experiments do not support the contention that the cells themselves change in their behaviour but are compatible with the first alternative that there is an increase in the lymphocyte trapping mechanism in the draining node.

It has been suggested that adjuvanticity, the property of an antigen itself or of some other substance which enables an immune response to result from the injection of an antigen (Dresser, 1961), may act through the stimulation of the division of an antigen-sensitive cell (ASC) (= immunocompetent cell?) in the presence of antigen (Dresser, 1968). Stimulation by an injected adjuvant, of the migration of a population of cells likely to be rich in ASC to a site in close proximity to an antigen trapping mechanism (Balfour and Humphrey, 1966; Humphrey, 1969; White, 1969) immediately prior to the initiation of division of ASC, would teleologically speaking be an optimal situation for the stimulation of an immune response. Thus adjuvanticity may be the cumulative effect of two *in vivo* effects, first, the stimulation of the localization of large numbers of ASC close to trapped antigen and second, the stimulation of the division of ASC. Alternatively, the adjuvant may stimulate the localization of ASC in a lymphoid organ and it might then be the physical environment in that organ, which initiates cell division rather than a direct action of the adjuvant. The well documented events of an immune response would then follow.

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