LYMPHATIC DRAINAGE AND ADJUVANT INDUCED ARTHRITIS IN RATS

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THE arthritic syndrome which develops in rats after intradermal injections of Freund's adjuvant (a fine suspension of dead mycobacteria in liquid paraffin), into certain sites has been previously described (Stoerk, Bielinski and Budzilovich, 1954 ; Pearson, 1956, 1959 ; Houssay and Frangione, 1961 ; Ward and Jones, 1962 ; Newbould, 1963). The most striking feature of this syndrome is the development of inflamed lesions called secondary lesions, remote from the injection site, after a delay of approximately 10 days. Waksman, Pearson and Sharp (1960) have suggested that the disease process represents a delayed hypersensitivity response to a disseminated antigen, probably derived from the tubercle bacillus but detailed knowledge of the steps involved is lacking.

It is known that intradermal injections into the foot-pad, tail or flank are superior to subcutaneous or intramuscular injections for producing the syndrome (Pearson, 1956 ; Houssay and Frangione, 1961 ; Ward and Jones, 1962), but the reasons for these differences have not been investigated. Since the intradermal route gives ready access to the lymphatic system (Hudack and McMaster, 1933) it might be supposed that the regional lymph nodes play a leading part in the development of the hypersensitivity. Ward and Jones (1962) provided valuable evidence to support this view by showing that removal of the depot within 2 hr. of injecting adjuvant into the tail prevented the development of secondary lesions. However, Waksman *et al.* (1960) failed to affect the development of the syndrome by removing the popliteal lymph node before or after injecting adjuvant into the foot pad. This procedure is effective in preventing the development of allergic encephalomyelitis which these authors consider to have many features in common with adjuvant induced arthritis.

In an attempt to understand more fully the reasons for the difference in efficacy of various injection procedures and the role of the lymphatic system in the development of the syndrome, changes in lymph nodes have been studied after injecting coloured adjuvant into various sites.

METHODS

Animals

Male, specific pathogen-free albino rats, Alderley Park strain I were used. They belonged to a colony-bred strain of rats of Wistar origin, and weighed approximately 200 g.

Production of arthritis

Attempts were made to produce the arthritic syndrome by injecting 0·05 ml. of a fine suspension of dead tubercle bacilli in liquid paraffin B.P. (5 mg./ml.) intradermally or subcu-

taneously into the middle of the right flank, or intradermally into the foot-pad or tail. Adjuvant coloured with the dye Fat Red 7B (Ciba Clayton Ltd.), 0·5 per cent w/v, was used when it was necessary to examine lymph nodes 15 days after injection. Tubercle bacilli were derived from human strains PN, DT and C which were grown for 8 weeks, killed by steam and dried in a vacuum oven. The development of the arthritic syndrome was followed by measuring the swellings in the hind feet across a sagittal section, with a micrometer (Newbould, 1963). The severity of secondary lesions was assessed in each rat as nil (0) ; mild (1) ; moderate (2) ; moderately severe (3), or severe (4). The figures in parentheses were used to give the total secondary lesion score in each group of rats.

Lymph nodes

Lymphatic pathways from injection sites in the tail, foot-pad and flank were revealed by injecting 0·05 ml. of Pontamine Sky Blue in water (4 per cent) or, so as to simulate the conditions used in the production of adjuvant-induced arthritis, 0·05 ml. of a fine suspension of dead tubercle bacilli in liquid paraffin B.P. (5 mg./ml.) coloured with the dye Fat Red 7B, 0·5 per cent w/v. The rats were killed 6 hr. after injection of Pontamine Sky Blue and 24 hr. after injection of coloured adjuvant, and the lymph nodes examined for the presence of dye. The colour of the lymph nodes was assessed as + (slight) ; ++ (moderate) or +++ (intense). In some experiments, lymph nodes were examined for colour and weighed 15 days after the injection of coloured adjuvant. The total lymph node weight, referred to in the section on results, is the sum of the weights of the popliteal, inguinal, axillary, para-aortic, and renal lymph nodes on both sides of the midline. These nodes were revealed as the most important in the studies with Pontamine Sky Blue. Lymph nodes were named following the nomenclature of Sanders and Florey (1940).

Injections into lymph nodes

Injections of 0·003 ml. of coloured adjuvant were made into the right inguinal lymph node or into the fat surrounding this node with an " Agla " micrometer syringe. The node was exposed by a ventral midline incision in animals anaesthetised with pentobarbitone.

RESULTS

Rats injected with adjuvant intradermally into the right hind foot-pad, tail, or right flank developed inflamed swellings on their hind feet from day 10 onwards (Fig. 1A, B, and C). Rats injected with adjuvant subcutaneously into the flank did not develop secondary lesions, hence the thickness of their hind feet remained constant (Fig. 1D).

The relationship of the lymphatic system to these injection sites was examined by injecting either Pontamine Sky Blue or adjuvant coloured with Fat Red. The nodes coloured in each group of 4 rats are shown in Table I and II. Both techniques showed that the sub-cutaneous route, which was unsatisfactory for producing secondary lesions, gave the lowest number of coloured nodes.

To examine simultaneously the relationship between the draining lymph nodes and the efficacy of various injection sites in the production of the arthritis syndrome, groups of 4 rats were injected with coloured adjuvant into the 4 sites used in the preliminary experiments. Fifteen days after injection the following details were recorded (Fig. 2) ; mean increase in thickness of the right and left hind foot, severity of secondary lesions ; body weight change from day 0 ; total number of coloured nodes and the lymph node weights. Lymph node weights were recorded because previously we had noted enlargement of the draining lymph nodes in rats with adjuvant-induced arthritis. Intradermal injections of coloured adjuvant into the right hind foot gave the greatest increase in thickness of the un-injected left hind foot, severe secondary lesions in all rats, a mean weight loss of 4 g., a

total of 9 coloured nodes and a striking increase in the mean total lymph node weight. Intradermal injections into the tail and flank were only slightly less effective than foot pad injections for producing the arthritic syndrome, but whilst injections into the tail stained a total of 11 lymph nodes and produced the greatest

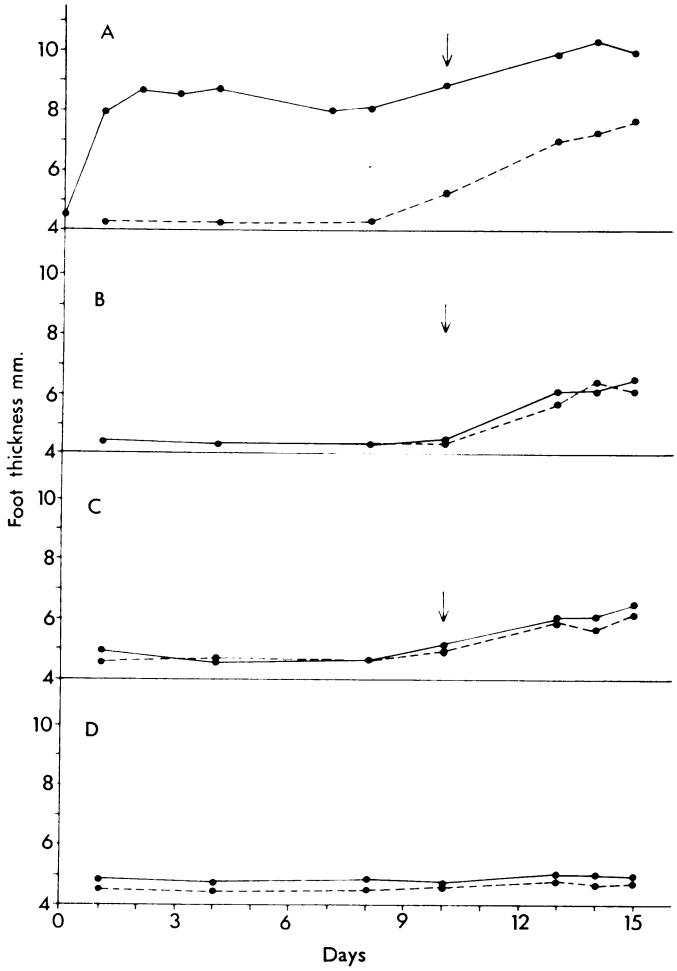

FIG. 1.—Changes in the thickness of both hind feet of rats injected with adjuvant, A : intradermally into the right hind foot ; B : intradermally into the tail ; C : intradermally into the middle of the right flank ; and D : subcutaneously into the middle of the right flank. Each point represents the mean foot thickness of 4 rats. ●————● thickness of the right hind foot ; ●– – – –● thickness of the left hind foot ; ↓ = day on which secondary lesions were first detected.

increase in the mean total lymph node weight, intradermal injections into the flank stained a total of only 4 nodes and produced only a moderate increase in the mean total lymph node weight. Subcutaneous injections into the middle of the right flank were generally ineffective for producing the arthritic syndrome. However, one rat, number 3 in this group, did develop mild secondary lesions. These were

TABLE I.—*Distribution of Pontamine Sky Blue 6 hr after Injection into Various Sites*

Injection site		Lymph nodes coloured with pontamine sky blue: Left side P	I	A	PA	R	IL	P.A.W.	Right side P	I	A	P.A.	R	IL	P.A.W.	Total number of coloured nodes
i.d. into the plantar surface of the right hind foot	1	..	..	..	..	..	..	..	+++	+	..	+++	++	..	..	21
	2	..	..	..	..	+	..	..	+++	++	+	+++	+	..	..	..
	3	..	..	..	..	++	..	..	+++	+	+	+++	++	..	..	...
	4	..	..	..	..	..	..	..	+++	++	+	+++	+++	..	..	..
i.d. into the tail	1	+++	..	..	++	+	+	..	..	++	+	+	+	..	..	25
	2	..	..	..	+++	++	..	..	..	..	..	+++	..	..	..	..
	3	+++	..	..	+++	+	..	+++	..	++	++	+++	++	..	..	..
	4	+++	..	..	++	++	..	..	+++	..	..	++	++	..	..	..
i.d. into the middle of the right flank	1	..	..	..	..	..	..	..	..	..	+	..	..	..	..	6
	2	..	..	..	..	..	..	..	..	+	+	..	..	..	..	..
	3	..	..	..	..	..	..	..	..	..	+	..	..	..	..	..
	4	..	..	..	..	..	..	..	..	+	+	..	..	...	..	..
s.c. into the middle of the right flank	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	3
	2	..	..	..	..	..	..	..	..	..	+	..	..	..	..	..
	3	..	..	..	..	..	..	..	..	..	+	..	..	..	..	..
	4	..	..	..	..	..	..	..	..	..	+	..	..	..	..	..

Colour of lymph glands
+ = slight.
++ = moderate
+++ = intense

P = Popliteal
I = Inguinal
A = Internal axillary
PA = Para aortic
R = Renal
IL = Iliac
P.A.W. = Posterior abdominal wall

TABLE II.—*Distribution of Adjuvant Coloured with Fat Red 24 hr after Injection into Various Sites*

Injection site	Rat	Lymph glands coloured with fat red: Left side P	I	A	PA	R	IL	P.A.W.	Right side P	I	A	PA	R	IL	P.A.W.	Total number of coloured glands
i.d. into the plantar surface of the right hind foot	1	..	..	..	+	..	..	..	++	..	..	++	..	..	..	12
	2	..	..	..	..	..	..	..	++	..	..	+	+	..	..	..
	3	..	..	..	..	..	..	..	++	..	..	+	+	...	..	..
	4	..	..	..	..	..	..	..	++	+	..	++	..	..	..	..
i.d. into the tail	1	+	..	..	+	..	..	..	..	..	..	+	..	..	..	11
	2	..	..	..	+	..	..	..	..	..	..	..	..	..	..	..
	3	+	++	..	..	..	..	..	..	+	..	+	..	..	..	..
	4	+	+	..	+	..	..	..	..	..	..	..	..	..	..	..
i.d. into the middle of the right flank	1	..	..	..	..	..	..	..	..	..	+	..	..	..	..	5
	2	..	..	..	..	..	..	..	..	..	+	..	..	..	..	..
	3	..	..	..	..	..	..	..	..	+	+	..	..	..	..	..
	4	..	..	..	..	..	..	..	..	..	+	..	..	..	..	..
s.c. into the middle of the right flank	1	..	..	..	..	..	..	..	..	..	..	..	..	..	..	0
	2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
	3	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
	4	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..

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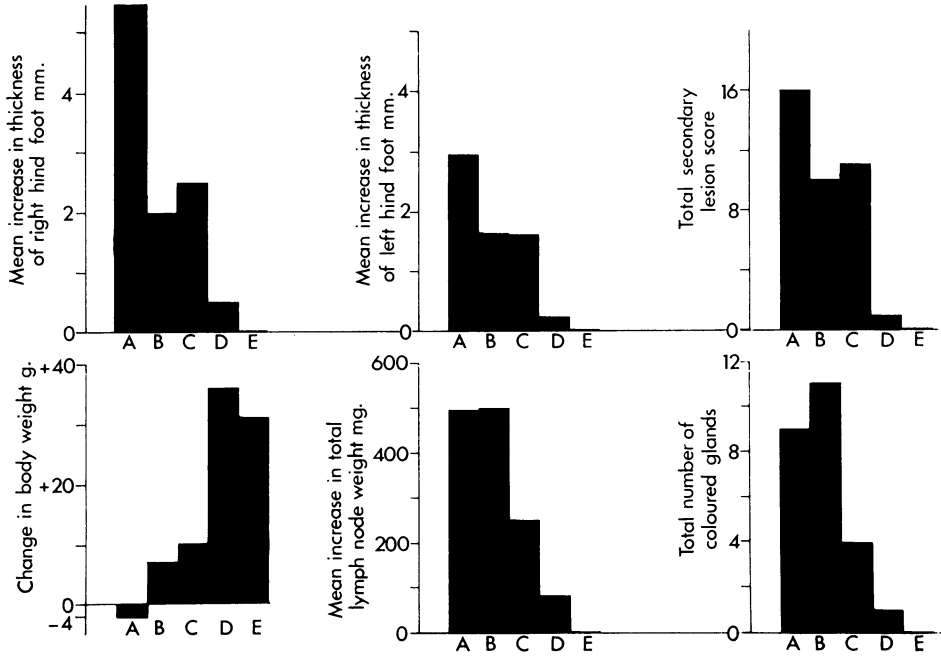

FIG. 2.—Comparison of results obtained 15 days after the injection of coloured adjuvant. A: intradermally into the right hind foot; B: intradermally into the tail; C: intradermally into the middle of the right flank; D: subcutaneously into the middle of the right flank; E: Un-injected controls. Each group contained 4 rats.

associated with a reduced weight gain, a striking increase in the total lymph node weight and one coloured gland (Table III).

The contribution of the weights of individual lymph nodes to the mean total lymph node weight used in the above comparisons is shown in Fig. 3. Each line is proportional in length to the mean increase in lymph node weight in each group of 4 rats, and when the line is broken it indicates that at least one node was coloured with the dye Fat Red. Intradermal injections of coloured adjuvant into the right hind-foot and tail resulted in an increase in weight of the inguinal, axillary, para-aortic, renal and popliteal nodes on both sides of the midline. Intradermal injections into the middle of the right flank gave a striking increase in weight of the internal axillary node but only marginal increases in the weights of the other nodes.

TABLE III.—*Data Obtained from 4 Rats 15 days after the Subcutaneous Injection of 0·05 ml. of Coloured Adjuvant*

	Rat			
	1	2	3	4
Secondary lesions	nil	nil	mild	nil
Weight change (g.)	+48	+52	+8	+39
Increase in total lymph node weight (mg.)	85	0	276	29
Number of glands coloured with Fat Red	0	0	1 (Internal axillary)	0

The glands which showed the greatest increase in weight were nearly always those in which coloured dye was found on at least one occasion.

As previously noted, only one of 4 rats developed secondary lesions following the subcutaneous injection of coloured adjuvant. The internal axillary node of this

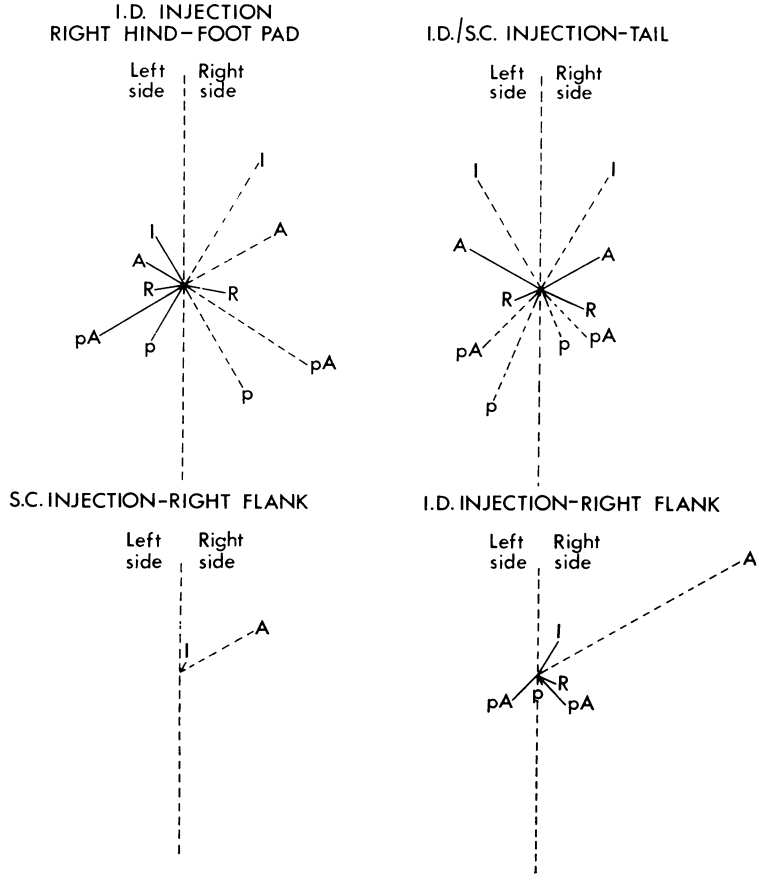

FIG. 3.—Mean increase in weight of individual lymph nodes 15 days after injecting a fine suspension of dead tubercle bacilli in coloured liquid paraffin into 4 different sites. The length of each line is proportional to the mean increase in lymph node weight observed in 4 rats. Broken lines indicate that at least one node was coloured. Key to lymph nodes: I = inguinal; A = axillary; R = renal; PA = para-aortic; P = popliteal.

rat was the only one to show a striking increase in weight and this was also the only node in which red dye was found.

These results indicated that if adjuvant gained access to the lymphatic system as evidenced by the colour or increase in weight of at least one lymph node, secondary lesions could be satisfactorily produced. To test this observation, 0·003 ml. of coloured adjuvant was injected into the right inguinal lymph node in one group of rats and into the fat surrounding the lymph node in a second group. All the rats injected with coloured adjuvant into the inguinal lymph node developed

TABLE IV.—*Secondary Lesions, Mean Increase in Weight of and Presence of Coloured Adjuvant in Individual Lymph Nodes 15 days after Injection of Coloured Adjuvant into the Right Inguinal Lymph Node and into the Fat Surrounding it.*

		Mean increase in weight of individual lymph nodes mg.									
		Right side					Left side				
Injection site	Secondary lesions	I	A	P	PA	R	I	A	P	PA	R
Right inguinal lymph node	Present in all rats	+171	+145	16	39	17	21	23	18	27	18
Fat surrounding right inguinal lymph node	nil	15	19	6	18	7	7	22	8	3	2

+ = coloured adjuvant present.

secondary lesions—(Table IV). The rats were killed 15 days after injection and the nodes examined and weighed. The right inguinal and axillary nodes of rats injected into the inguinal lymph node were stained red and showed a striking increase in weight. None of the controls developed secondary lesions. Furthermore, there was no appreciable increase in lymph node weight and the red coloured adjuvant could be seen as a discrete globule which had remained at the site of injection.

DISCUSSION

These results show that the pre-requisite for the development of secondary lesions in rats injected with adjuvant is the passage of some of the injected material into the lymphatic system. The role, if any, of the lymph nodes in the pathogenesis of the syndrome cannot be determined from this series of experiments. Previously it has been shown that removal of the popliteal lymph node 5 days before or 7 days after injection into the foot pad did not affect either the rate with which arthritis appeared or its severity (Waksman *et al.*, 1960). However, it has now been shown that after injection into the foot pad, adjuvant is disseminated beyond the popliteal node. It therefore seems likely that more lymph nodes would have to be removed before it could be ascertained that none was contributing to the development of secondary lesions. Experiments to check this possibility are now in progress.

SUMMARY

The relationship between draining lymph nodes and the efficacy of various injection sites in the production of the arthritis induced in rats by mycobacterial adjuvant has been studied using coloured adjuvant. The production of inflamed lesions remote from the injection site appears to depend on the ease with which the injected material gains access to the lymphatic system. Coloured adjuvant was found in at least one lymph node in all rats which developed secondary lesions. It was not found in the lymph nodes of rats injected with coloured adjuvant which did not develop secondary lesions. These observations were confirmed by showing that secondary lesions could be produced by injecting small volumes of adjuvant directly into the inguinal lymph node. Secondary lesions were not produced when a similar volume was injected into the fat surrounding the inguinal lymph node.

It has been shown that coloured adjuvant can be detected in lymph nodes other than the popliteal after injection into the foot pad.

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REFERENCES

HOUSSAY, R. H., FRANGIONE, B.—(1961) In 'Atti Del X Congresso della lega internazionale contro il reumatismo', Volume II, Turin (Tipografia Edizioni Minerva Medica), p. 950.

HUDACK, S. S., MCMASTER, P. D.—(1933) *J. exp. Med.*, **57**, 751.

NEWBOULD, B. B.—(1963) *Brit. J. Pharmacol.*, **21**, 127.

PEARSON, C. M.—(1956) *Proc. Soc. exp. Biol. N.Y.*, **91**, 95.—(1959) in 'Mechanisms of Hypersensitivity'. Ed. J. H. Shaffer, G. A. Lo Grippo and M. W. Chase. Boston (Little, Brown and Co.), p. 647.

STOERK, H. C., BIELINSKI, T. C. AND BUDZILOVICH, T.—(1954) *Am. J. Path.*, **30**, 616.

SANDERS, A. G. AND FLOREY, H. W.—(1940) *Brit. J. exp. Path.*, **21**, 275.

WAKSMAN, B. H., PEARSON, C. M. AND SHARP, J. T.—(1960) *J. Immunol.*, **85**, 403.

WARD, J. R. AND JONES, R. S.—(1962) *Arthritis and Rheum.*, **5**, 557.