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Antigen Localization in Lymphopenic States

II. FURTHER STUDIES ON WHOLE BODY X-IRRADIATION

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Summary. The gross and microscopic distribution of ^{125}I polymerized flagellin from *Salmonella adelaide* was studied in adult rats at various times following 800 r whole body X-irradiation. Injections of radioactive antigen were made in both hind footpads, and the popliteal lymph nodes were excised for autoradiographic study 1 day later. This dose of irradiation caused a progressive decline in the ability of lymphoid follicles of popliteal nodes to capture and retain antigen. Irradiation had no detectable effect upon antigen uptake by whole lymph nodes or upon the number of grains overlying the phagocytic cells of the medullary sinuses of popliteal nodes.

Various substances capable of restoring follicular antigen uptake in the irradiated rat were studied by means of injecting the test substance into one hind footpad 1 hour prior to the injection of antigen into both feet. The distribution of antigen in each popliteal node was compared, each animal thus acting as its own control. It was found that 0·01 ml of specific anti-flagellar immune serum, or 0·25 ml of normal adult rat serum significantly improved follicular antigen uptake when tested ten days after irradiation. Foetal calf serum, homologous lymphocytes, and the media from pooled concentrated lymphocyte cultures were without demonstrable effects when given by regional injection. Shielding of the popliteal nodes at the time of irradiation improved follicular antigen uptake, whereas shielding of the femoral bone marrow and appendix was ineffective. No agent found capable of improving follicular antigen capture in the irradiated rat significantly altered footpad retention of antigen, whole organ counts of the popliteal nodes, or antigen localization in the phagocytic cells of the lymph node medulla.

The results favour the interpretation that the follicular antigen trapping mechanism is extremely sensitive to changes in levels of opsonins; that substances present in normal adult rat serum act as 'follicular opsonins'; that these substances decline exponentially following irradiation; and that these substances are secreted by small lymphocytes or their progeny.

INTRODUCTION

In the preceding paper (Williams, 1966) evidence was presented indicating that lymphocyte depletion produced by chronic thoracic duct drainage resulted in a decreased ability of lymphoid follicles to take up polymerized flagellin from *Salmonella adelaide*. Both autogenous lymphocyte infusions and administration of small amounts of specific anti-flagellar immune serum restored the follicular concentrating capacity towards normal.

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These findings suggested that lymphocytes may elaborate substances with opsonic properties which are important in determining the distribution of antigen within lymphoid tissue. If this hypothesis were correct, then one would expect to find depressed follicular antigen uptake in other lymphopenic states. The irradiated rat provides a logical model for further studies, particularly in view of the findings of others that irradiation reduced the levels of 'natural antibodies' in serum (Talmage, 1955; Kornfeld and Miller, 1960; Kornfeld, Hammond and Miller, 1960).

However, previous studies in our laboratory (Jaroslow and Nossal, 1966a) have indicated that there is little impairment in the ability of lymph node follicles to capture antigen when antigen is given shortly after irradiation. These studies have shown that the web of reticular cell fibrils in follicles responsible for antigen trapping is relatively radio-resistant, 8000–16,000 r being required to destroy it completely. They indicated, however, that the follicular mechanism was not entirely intact functionally 1 day after as little as 450 r. The present experiments were designed to extend these findings utilizing quantitative autoradiographic techniques. Particular attention was given to the possibility that defects in follicular antigen trapping might be delayed following irradiation, reflecting the decay of existing opsonins.

We now report that 800 r whole body irradiation produces a progressive loss in the ability of follicles to capture polymerized flagellin, and that normal rat serum as well as small quantities of antibody are capable of restoring follicular uptake towards normal, even in the heavily irradiated rat.

MATERIALS AND METHODS

Animals

Outbred Wistar rats aged from 12 to 14 weeks were used. In the initial experiments irradiated rats were fed water containing 1 mg/ml of tetracycline. This program was not successful in reducing mortality from irradiation, and the last three groups of irradiated rats were injected twice daily after X-ray with 5 mg of streptomycin and 2000 units of penicillin. Cages were changed once a day. In all, a total of sixty-one rats survived irradiation, providing a total of 122 popliteal nodes for quantitative study.

X-Irradiation

Rats were placed in a Perspex box, surrounded on four sides with 8 cm of packing having the radiation-absorbing characteristics of body tissues. This unit was placed on a lead plate, 2·5 cm thick, and exposed to a vertical beam. Radiation was carried out at 235 kV, 15 mA, half-valve layer of 1·0 mm Cu, at a dose rate of 68 r/min. The standard dose given was 800 r, which represents an LD_{45} at 11 days in this strain.

Antigen

Polymerized flagellin was used throughout in a dose of 30–60 μg, injected into both hind footpads.

Preparation of iodinated polymerized flagellin and autoradiographic technique

These procedures were carried out as described in the preceding paper (Williams, 1966).

Preparation of antiflagellar immune serum

Ten adult rats were bled 14 days following a secondary challenge with polymerized flagellin. Sera were pooled and aliquots were kept at $-40°$. The immobilization titre of the pooled serum was 1: 400.

Shielding procedures

An operative procedure was devised for the exclusive shielding of bone marrow. Anaesthetized animals were placed prone and the mid-shaft of the femur was exposed by means of a vertical incision placed over the linea aspera. The muscle surrounding the femur was dissected free from the bone leaving the periosteum intact. A strip of lead 1 cm wide by 2 cm long and 2 mm thick was placed around the bone and tightened to form a cylinder. The lead strips had been previously buffed to remove oxide and autoclaved. The muscle was sutured over the cylindrical piece of lead and the skin was closed with clips. Initially the lead was removed shortly after irradiation, but subsequent experiments showed that animals tolerated this large foreign body for a 10-day period without sepsis. When irradiation was carried out on an anaesthetized animal in the prone position no major lymphoid areas and very little other tissue was shielded by the lead encasing the mid-shaft of the femur.

The caecal apex, analogous to the appendix, and the mesenteric lymph node at the ileocaecal junction were shielded by anaesthetizing rats with Nembutal and placing these structures wrapped in sterile gauze within a 4 mm thick tunnel of lead. The tunnel was constructed in two parts such that the tissues could be positioned inside under direct vision and then the top thickness of lead was applied. This method provided excellent shielding of the appendix, but the efficiency of shielding structures such as the ileocaecal lymph node which lay more proximally in the tunnel was questionable.

The popliteal lymph nodes were shielded simply by placing the hind legs inside 4-mm thick lead cylinders 2 cm long at a level that overlay the popliteal fossae. Bone marrow is also shielded in this procedure, and the area of the groin may be protected to a slight degree.

Collection and infusion of lymphocytes

Rats were cannulated and restrained in order to collect thoracic duct lymph as outlined in the preceding paper (Williams, 1966). Lymph was collected for an 18-hour period in an excess of Eisen's balanced salt solution containing antibiotics and heparin but which did not contain any serum. At the end of this collection the cells were washed three times in the cold, resuspended in 10 ml of fluid and kept at 4°. Following irradiation the rats were restrained again and the lymphocytes infused intravenously during a 2-hour period.

Lymphocyte cultures were prepared from thoracic duct lymph and cells were pooled to give a concentration of 2×10^9 cells in 2 ml of medium 199 containing 20 per cent foetal calf serum. These cultures were incubated for 3 hours at 37°. Cell viability as judged by the uptake of eosin dropped to 60 per cent at the end of this period. Both the cellular fraction and the supernatant fluid were tested for their effect on follicular antigen capture in the irradiated rat. The purpose of this technique was to obtain concentrated crude fractions rich in lymphocyte products.

Quantitation of autoradiographic data

The assessment of the follicular concentration of antigen, the medullary concentration of antigen, and the follicular cross-sectional amount of antigen was made as presented in the preceding paper (Williams, 1966).

Quantitation of mean follicular cross-sectional area

In addition to determining the mean area of the three largest follicles on section, camera lucida drawings on bond paper were made of 60-day exposures of autoradiographic

sections of popliteal nodes. Those areas representing follicles were invariably sufficiently heavily labelled to be traced easily. All slides were projected at the same magnification, and follicular size was assessed from the mean weights of all follicular areas cut from the drawings. It should be realized that follicular size measurements are subject to variation induced by sectioning in which some follicles are cut in the centre and others at the periphery. The units of size used are arbitrary and relative.

RESULTS

The overall plan of the first series of experiments was to determine the time relationships involved in the decline of normal antigen processing following irradiation. Antigen was injected into the hind footpads at various times after X-irradiation and rats were killed 24 hours later. The regional lymph nodes were excised and assessed for the parameters of follicular concentration of antigen, medullary concentration of antigen, follicular size and follicular cross-sectional amount of antigen. Some animals studied 11 days after irradiation displayed lymph nodes composed almost exclusively of reticular cells rendering identification of follicles difficult on routine sections. However, if antigen labelled with over 100 μc of ^{125}I were injected, sufficient activity persisted for follicular remnants to be obvious on autoradiographic sections exposed 60 days.

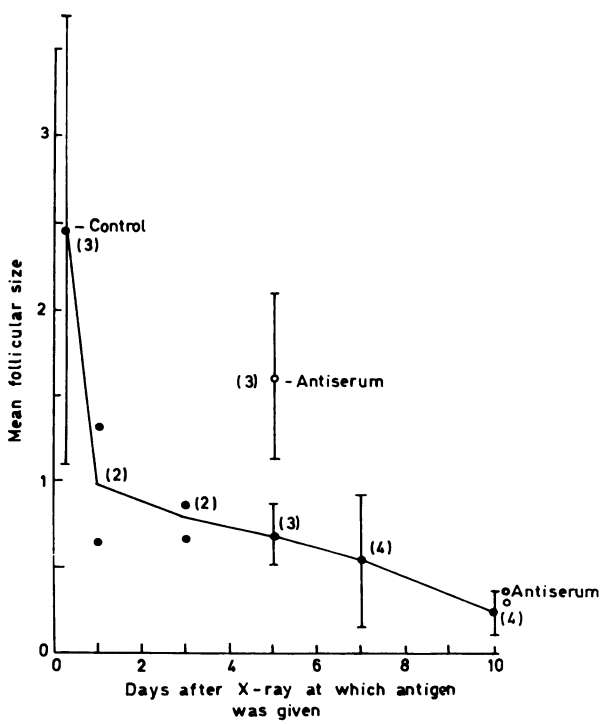


FIG. 1. Results of camera lucida tracings of follicular antigen trapping at various times following 800 r whole body X-irradiation. The units of follicular size are relative. The number of rats per point is given by the number in parentheses opposite each point. Vertical bars indicate standard deviation.

THE EFFECT OF IRRADIATION ON FOLLICULAR SIZE

The progressive contraction of follicular size that occurred following 800 r is shown by Fig. 1. There was a sharp decrease in cross-sectional area at 1 day following 800 r and the size at 10 days was almost one-tenth that of normal animals. These data confirm quantitatively the subjective impression of Jaroslow and Nossal (1966b) following a lower X-ray dose. Histological study of lymph nodes following X-ray revealed absence of lymphocytes. The follicular remnant was composed almost exclusively of reticular cells and their processes. However, the contraction in the area taking up antigen does not appear to be due entirely to the loss of lymphocytes as footpad injections of 0·01 ml of standard antiflagellar immune serum in 0·1 ml saline substantially increased the mean cross-sectional area taking up antigen when given 5 days after irradiation. Ten days after irradiation, immune serum had lost this effect.

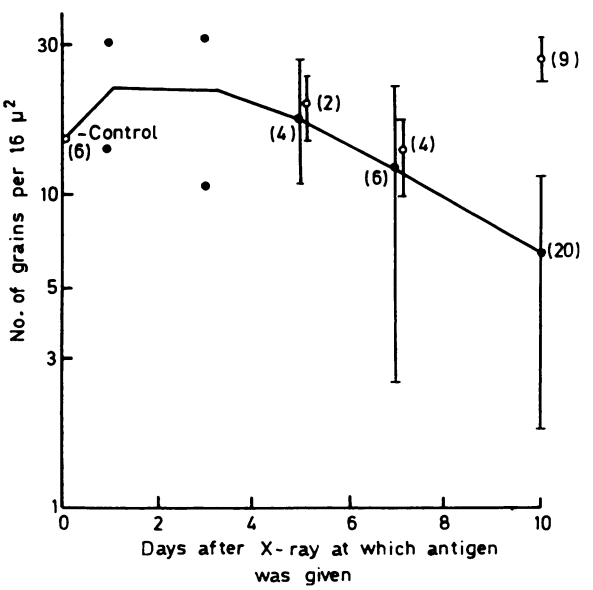


FIG. 2. Quantitative grain counts overlying the follicles of popliteal lymph nodes 24 hours after antigen injection at various times following 800 r whole body X-irradiation. In this and subsequent figures, the number of rats per point is given by the number in parentheses opposite each point. Vertical bars represent standard deviations. Antigen, polymerized flagellin, dosage 30–60 μg. Different dosages and batches of antigen were standardized by normalizing control values for follicular concentration. ●, irradiated; ○, immune serum.

THE EFFECT OF RADIATION ON FOLLICULAR ANTIGEN-CONCENTRATING ABILITY

The changes in follicular concentration of antigen occurring when antigen was injected at various times after 800 r of whole body irradiation are plotted in Fig. 2. Shortly after irradiation antigen was concentrated to at least normal levels by the follicles. After the 5th day there was a decline in follicular concentrating capacity, the considerable variation in levels resulting from one animal in four having normal values. An eye-fit straight line between the 5-, 7- and 10-day mean concentrations plotted semi-logarithmically yields a slope for the half life of the decline in antigen concentrating capacity of 3·4 days. The effects of footpad injections of 0·01 ml of the standard antiflagellar immune

serum in 0·1 ml saline on follicular concentration of antigen were minimal until the tenth day after radiation. At this time point immune serum injected into one footpad significantly increased the concentration of antigen in follicles in the ipsilateral but not the contralateral popliteal lymph nodes (Table 1).

THE EFFECT OF RADIATION OF THE TOTAL AMOUNT OF ANTIGEN CONTAINED IN LYMPHOID FOLLICLES

An estimate of the amount of antigen contained in follicles is provided by counting the number of grains contained within the three largest and most heavily labelled follicles in each lymph node section and determining the mean value. Fig. 3 demonstrates the decline in mean cross-sectional follicular amount of antigen 1 day after injection when antigen

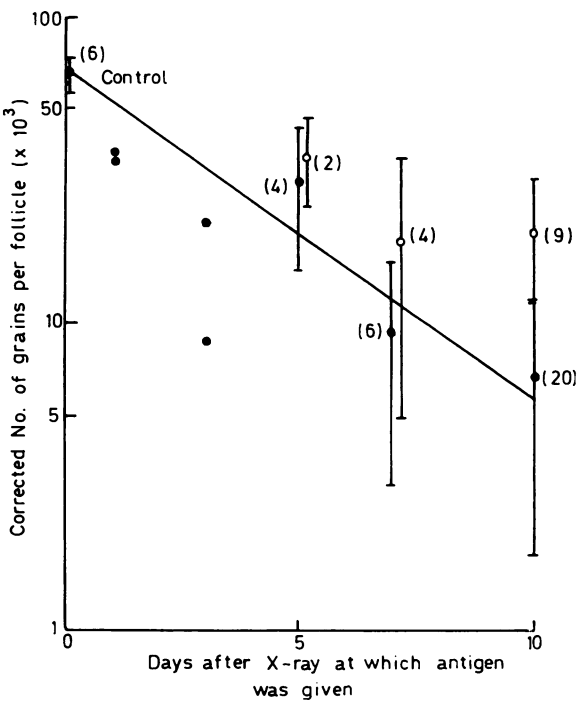


FIG. 3. Quantitative grain counts overlying follicles × follicular area, providing an estimate of the amount of antigen present in an average follicular cross-section 24 hours after injection at various times following 800 r whole-body X-irradiation. Half-life = 2·8 days; ●, irradiated; ○, immune serum.

was given at various times after irradiation. The amount of antigen in follicular areas declined soon after X-irradiation and fell to levels one-tenth of control values 10 days after irradiation. The time after irradiation required for follicular uptake of antigen to be halved is estimated to be 2·8 days. Standard 0·01 ml injections of immune serum significantly increased the average cross-sectional amount of antigen in follicles when antigen was 10 given days after irradiation. This difference is best demonstrated utilizing unilateral footpad injections of serum and comparing the uptake of antigen by ipsilateral and contralateral nodes (Table 1).

TABLE 1

THE EFFECTS OF REGIONAL INJECTIONS OF SERA AND LYMPHOCYTES AND OF SHIELDING PROCEDURES ON THE DISTRIBUTION OF POLYMERIZED FLAGELLIN 10 DAYS AFTER 800 r X-IRRADIATION

Treatment groups of irradiated rats	No. rats	Follicular concentration* (grains/16 μ^2)			Medullary concentration (grains/16 μ^2)			Follicular amount (grains/follicular cross-section)		
		Mean values	Mean difference ± S.E.	P	Mean	Mean difference ± S.E.	P	Mean	Mean difference ± S.E.	P
1. Unilateral injection of antiflagellar immune serum† *vs.* Contralateral injection of equal volume of saline	9	25·3 5·24	20·1 ± 2·47	< 0·01	10·1 15·5	−5·4 ± 3·8	n.s.	1760 318	1442 ± 368	< 0·01
2. Unilateral injection of fresh adult rat serum, 0·25 ml *vs.* Contralateral injection of foetal calf serum, 0·25 ml	4	10·4 4·75	5·6 ± 0·48	< 0·01	27·6 32·6	−5·1 ± 14·0	n.s.	1187 211	976 ± 334	< 0·02
3. Popliteal shielded *vs.* Non-shielded	4 4	38·5 26·7	11·8 ± 9·0	n.s.	13·2 11·9	1·3 ± 5·0	n.s.	11378 1448	9330 ± 3340	< 0·05
4. Popliteal shielded *vs.* Appendix shielded	4 6	13·7 2·58	11·2 ± 2·65	0·01	7·05 7·05	0	n.s.	5890 1230	4660 ± 1370	< 0·01
5. Unilateral injections of $7·3 + 10^8$ homologous lymphocytes *vs.* Contralateral injections of medium 199	4	7·36 5·91	1·45 ± 0·87	n.s.	—	—	—	755 795	−40 ± 172	n.s.

n.s. = Not significant.

* Units of concentration and amount represent direct grain counts. As such they are valid for comparisons within a treatment group, but they are not corrected for differences in exposure time between groups 1–5.

† 0·01 ml serum, titre 1:400 in 0·1 ml saline.

THE EFFECTS OF IRRADIATION ON MEDULLARY CONCENTRATION OF ANTIGEN

The concentration of antigen overlying the phagocytic cells of the medulla of lymph nodes was unaffecteed by irradiation (Fig. 4). If anything the concentration of antigen in the medulla 24 hours after injection was increased following irradiation. As counts of the medulla often included areas adjacent to the sinuses a proportion of antigen counted microscopically may simply have represented antigen in transit through these sinuses and not on or within phagocytic cells. Thus the state of flux of antigen may be quite pertinent to the amount of antigen counted in the medullary areas.

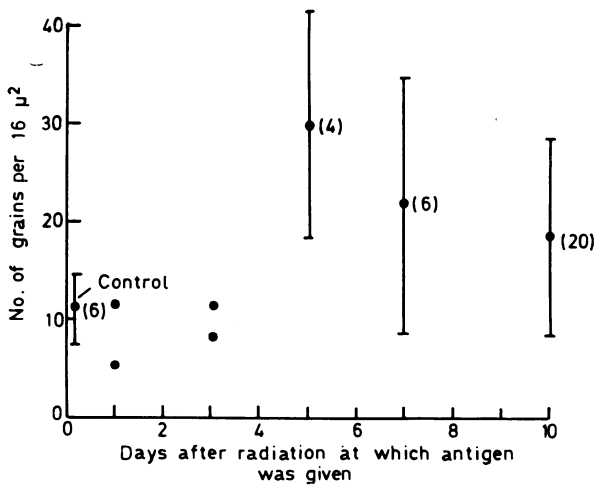
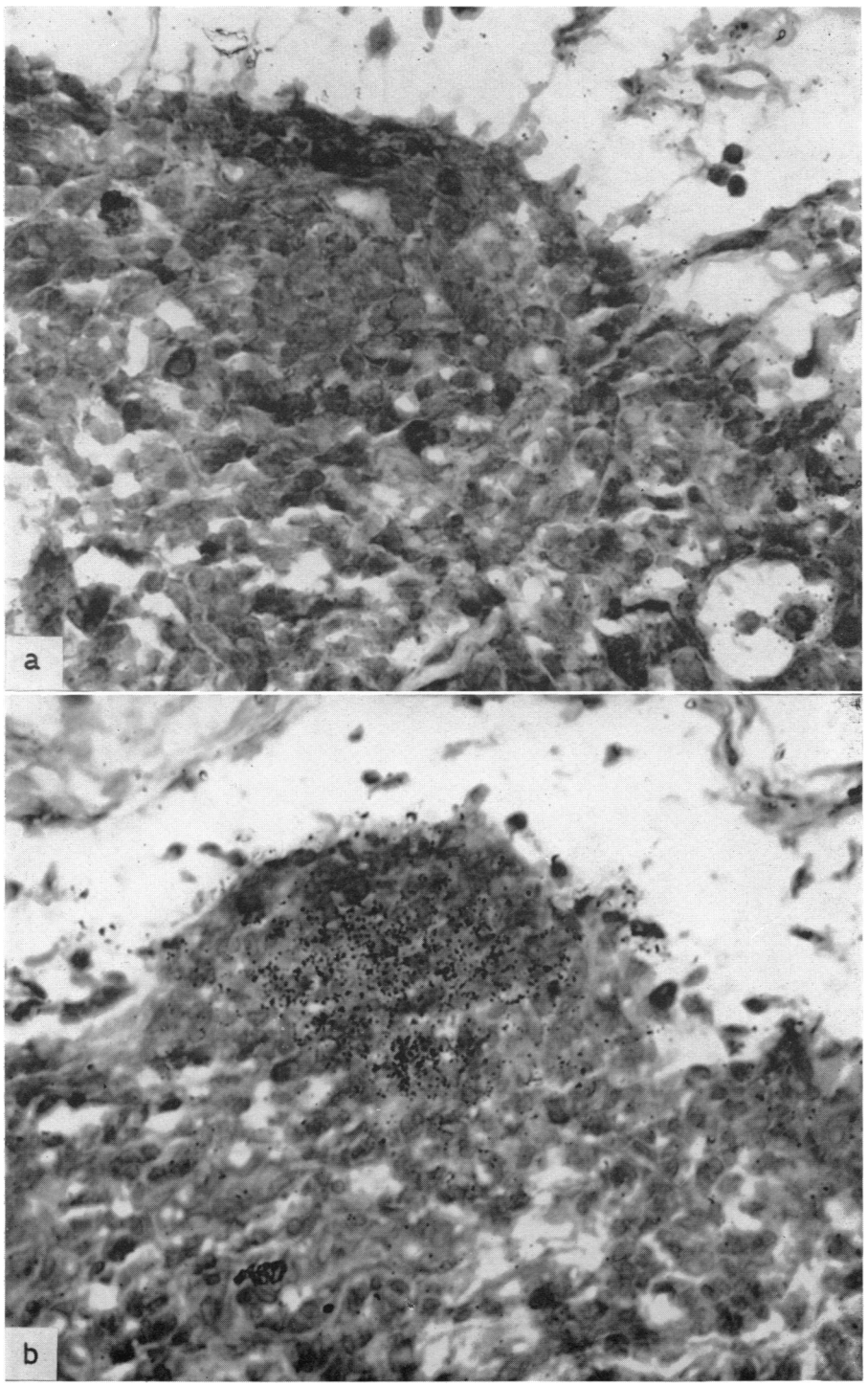


FIG. 4. Quantitative grain counts overlying the medulla of popliteal nodes 24 hours after antigen injection at various times following 800 r whole-body X-irradiation.

THE EFFECTS OF IRRADIATION ON ANTIGEN RETAINED AT THE SITE OF INJECTION AND ON THE AMOUNT OF ANTIGEN CONTAINED IN THE POPLITEAL NODE

In order to evaluate the degree of absorption of antigen from the footpad, the feet of twenty-eight animals were removed at the time of killing and counted in a uniform manner using a scintillation probe (Fig. 5). Antigen in a dose of 30 μg given 1 day after irradiation was poorly absorbed, 50 per cent remaining in the feet 24 hours after injection. By contrast, normal animals, and irradiated animals given antigen 7 and 10 days following irradiation contained just 25 per cent of the identical dose in the injection site. Thus whole-body X-irradiation appeared to have a transient effect, delaying absorption of this large protein from the footpad, but rats recovered from this effect 7 days after irradiation. However, as 25 per cent of the injected dose persisted in the feet, antigen must be assumed to be still in transit to and through regional nodes 24 hours after footpad injections.

FIG. 6. The effect of normal serum on follicular antigen-uptake 10 days after 800 r whole body irradiation. Representative follicles from right and left popliteal nodes from the same animals removed 24 hours after injection. (a) Side injected with 0·25 ml of foetal calf serum. (b) Side injected with 0·25 ml of normal adult rat serum. Polymerized flagellin, 30 μg labelled with 300 μc ^{125}I was injected into each hind footpad 1 hour after the injection of serum. Exposure time; 24 hours. × 250. Note the marked contraction of follicular size, absence of small lymphocytes, and concentration of grains in the follicle from the lymph node ipsilateral to the injection of normal serum. This exposure time was optimal for performing grain counts.



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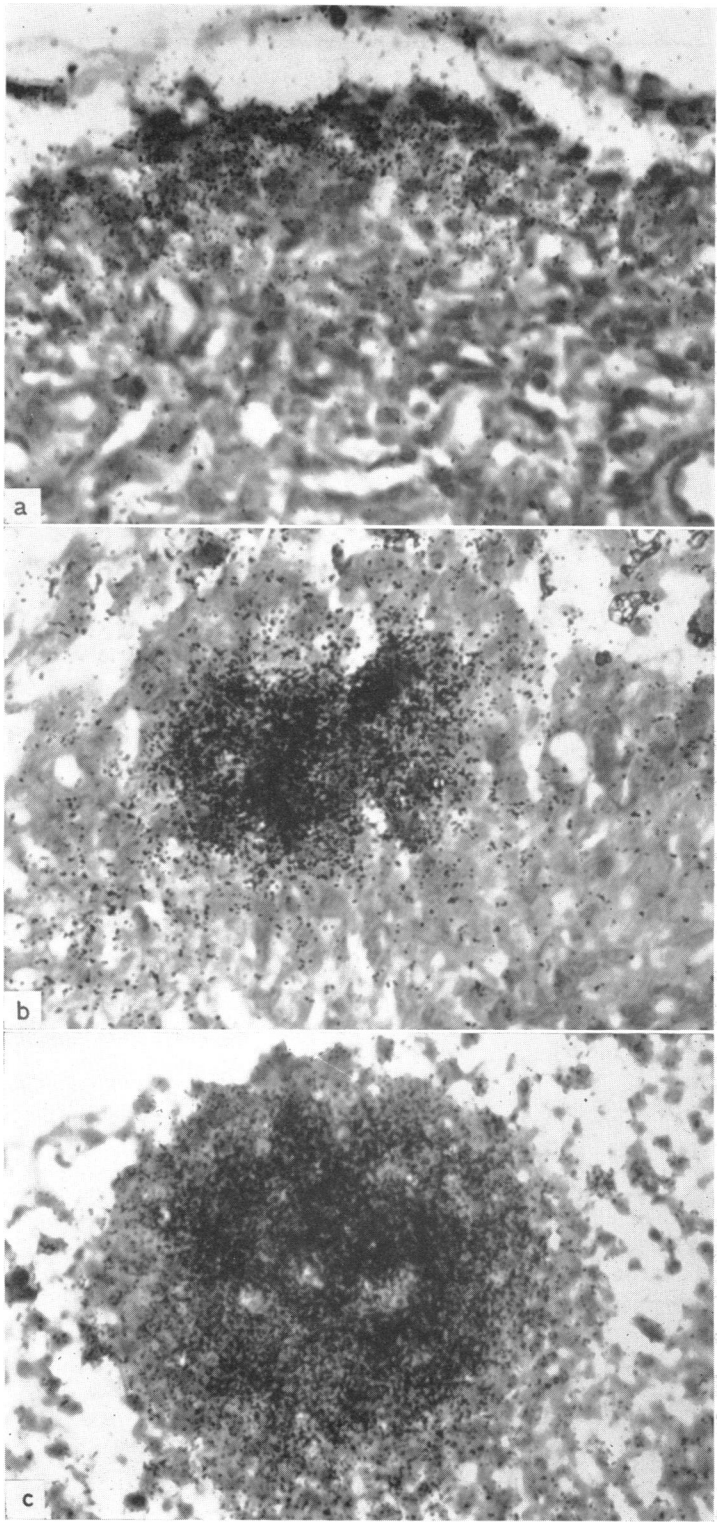
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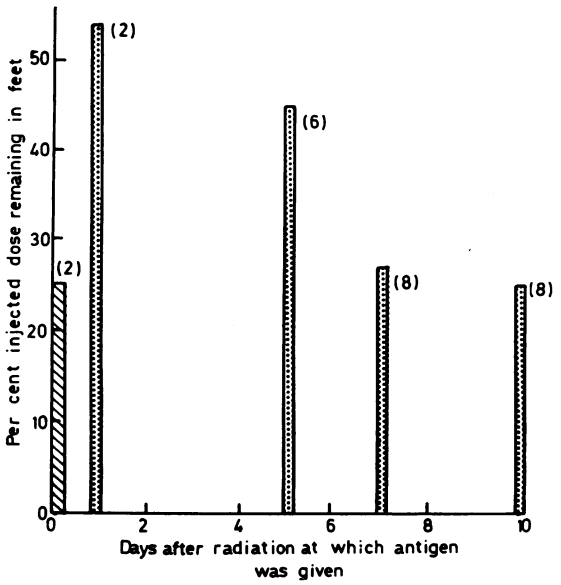

FIG. 5. Radioactivity counts of the feet 24 hours after antigen injection at various times following 800 r whole-body X-irradiation. The number of rats per point is given by the number at the top of each bar on the graph. Values for control animals are given at day zero.

Whole organ counts of the popliteal nodes were performed in all of the sixty-one animals in this study. Irradiation was found to have no appreciable effect on the amount of antigen contained in the node as a whole 24 hours after injection.

RESTORATION OF FOLLICULAR ANTIGEN-UPTAKE IN IRRADIATED ANIMALS

The plan of restoration experiments was to test the effects of regional injections of immune or normal adult rat serum or of shielding specific structures during irradiation, on the follicular uptake of antigen 10 days after irradiation. Whenever possible, comparisons were made between the two extremities of the same animal or between animals receiving the same dose and batch of iodinated antigen. When comparisons were made between groups of animals that had received different batches of antigen, follicular concentration was standardized by normalizing control values. The results for the various procedures tested for restorative activity are listed in Table 1, and representative photomicrographs are shown in Figs. 6 and 7.

FIG. 7. The effect of popliteal lymph node shielding or immune serum on follicular antigen-uptake 10 days after 800 r whole body irradiation. (a) and (b) are representative follicles from the right and left popliteal lymph nodes of the same animal removed 24 hours after antigen injection.

(a) Side injected with 0·1 ml of saline 1 hour prior to antigen. × 200.

(b) Side injected with 0·01 ml of standard immune serum in 0·1 ml saline 1 hour prior to antigen injection. × 200.

(c) Follicle from an animal shielded in the popliteal area at the time of irradiation. × 80.

Antigen, polymerized flagellin, 30 μg, labelled with 200 μc of ^{125}I. Exposure time, 3 days. There is a clear cut difference in the amount of antigen contained in the follicle that was exposed to the regional injection of immune serum. The architecture of the follicle was well preserved in the shielded node, and antigen is found present in high concentration. The scattering of grains in the zone of cortex just deep to the cortical sinus (a) was seen quite often in the irradiated rat. This particular exposure time was too long for obtaining accurate grain counts, but demonstrates the magnitude of the differences obtained using restorative agents.

A small amount of specific anti-flagellar immune serum significantly improved the ability of follicles to take up and concentrate antigen. The follicles from popliteal nodes ipsilateral to the foot injected with 0·01 ml of immune serum captured an average of five times more antigen than the contralateral side, and this difference was highly significant ($P<0·01$). Normal rat serum in volumes twenty-five times that of immune serum also increased the ability of the follicles to take up antigen significantly when compared directly with the same volume of foetal calf serum injected into the opposite extremity (Fig. 6a and b). Shielding of the popliteal nodes was done bilaterally, and the results of such shielding in four animals revealed significant increases in the amount of antigen taken up by follicles compared to groups of unshielded, appendix-shielded and bone marrow-shielded animals (Fig. 7a and c). The latter two shielding procedures, unilateral footpad injections of either $7·3\times10^8$ homologous thoracic duct lymphocytes (60 per cent viable), and injections of 0·5 ml aliquots from 2 ml of supernatant fluid from a 3-hour culture of 20×10^9 thoracic duct lymphocytes were without appreciable effects on follicular function. All animals shielded at the time of irradiation survived the 11-day test period in contrast to the 45 per cent mortality of non-shielded irradiated rats.

None of the agents tested significantly altered the concentration of antigen within the medulla of the lymph nodes. Likewise neither normal nor immune serum affected footpad retention of antigen, or the amount of antigen captured by the entire popliteal node in the thirteen animals tested. Thus while both normal and immune serum caused a five-fold increase in the amount of antigen taken up by follicles neither agent produced consistent effects on the gross organ distribution of antigen.

THE EFFECT OF INTRAVENOUS INFUSIONS OF AUTOLOGOUS LYMPHOCYTES ON FOLLICULAR LOCALIZATION OF ANTIGEN IN IRRADIATED RATS

Inbred rats were unavailable for this study, and as a result a limited experiment was conducted to test the ability of thoracic duct lymphocytes to restore follicular localization of antigen in irradiated rats. Two rats were subjected to thoracic duct cannulation and their lymphocytes were collected for 1 day. The rats then received 800 r whole body irradiation, and 2 hours later, the first received 8×10^8 and the second 2×10^8 of its own lymphocytes intravenously. Each was then tested for its capacity for follicular antigen localization 10 days later. The results are summarized in Table 2. The rat receiving 8×10^8 autochthonous lymphocytes was found to take up antigen at least as well as non-irradiated

TABLE 2

Treatment group	Follicular concentration (grains/16 μ^2)	Medullary concentration (grains/16 μ^2)	Follicular Size ($\times$ 16 μ^2)	Follicular amount (grains/follicular cross-section)
(1) Non-irradiated control	4·94	5·3	297	1460
(2) 8×10^8 lymphocytes re-infused	11·9	7·8	206	2620
(3) 2×10^8 lymphocytes re-infused	5·64	13·3	76	470
(4) Irradiated controls (four)	$4·17\pm3·14$	$8·5\pm2·33$	$76\pm16·1$	550 ± 480
Significance of difference between (2) and (4)	Difference = 11·9–4·17 = 7·13 $\tau = \frac{\text{difference}}{\text{S.D.}} = \frac{7·13}{3·14} = 2·27$ $P = 0·02$	Difference = 206–76 = 130 $\tau = 130/16·1 = 8·08$ $P \ll 0·001$	Difference = 2620–550 = 2070 $\tau = 2070/480 = 4·82$ $P \ll 0·001$	

animals whereas the other rat which received four times fewer cells was in all respects similar to irradiated controls. The magnitude of the differences between the animal receiving 8×10^8 cells post-irradiation and the group of irradiated animals in this experiment suggests that this degree of difference is most unlikely on the basis of chance alone.

DISCUSSION

The results presented in this and in the preceding paper (Williams, 1966) indicate that the small lymphocyte may play an important role in determining the distribution of polymerized flagellin in regional lymph nodes footpad injections. Furthermore, in the lymphopenic rat the association between the ability of normal serum, immune serum and lymphocyte infusions to improve follicular antigen-uptake suggests that lymphocytes may secrete substances found in normal serum with opsonic properties similar to those of specific antibody. However, as drastic procedures were used to induce specialized changes in antigen distribution, and as the physiology of subcutaneous absorption is complex, conclusions must be made with caution.

The initial question of importance relates to the significance of the defect observed in antigen-processing following either thoracic duct drainage or irradiation. What, in fact, is measured by a decline in the ability of follicles in regional lymph nodes to capture antigen? Previous work has demonstrated that follicular antigen-capture by regional nodes is enhanced by either active or passive immunization (Nossal, Ada, Austin and Pye, 1965). In the unimmunized animal follicular antigen-uptake correlates roughly with the potency of a given antigen. Only well phagocytosed antigens are found in high concentration in follicles following injection (Ada, Nossal, Pye and Abbot, 1964). It thus appears likely that high levels of opsonins are required to localize antigens in follicles, whereas medullary macrophages may require much lower concentrations. Consequently, if opsonic decay occurs following irradiation, the system requiring the higher levels of opsonic factors for antigen capture might be expected to exhibit a greater decline in this capturing ability.

In addition, another possible explanation exists for the variability and lack of sensitivity observed in antigen-capture by medullary sinus-macrophages following irradiation. These studies have confirmed earlier work (Ada *et al.*, 1964) showing that less than 0·1 per cent of the injected dose of antigen is present in the popliteal nodes 24 hours after injection. As 25 per cent of the injected dose persists in the feet at this time (Fig. 5) considerable amounts of antigen must be assumed to be in the transit through afferent lymphatics and the sinusoids of the lymph node medulla. As the amount of antigen in transit may well exceed that captured by macrophages at this time point, changes in the uptake of antigen by sinus macrophages might go undetected. Grains emerging from the adjacent sinusoids can not be clearly distinguished from grains originating from cells. Thus follicular antigen-uptake may reflect opsonic levels more accurately because the follicles are removed from the mainstream of lymphatic flow through the lymph node (Yoffey and Courtice, 1956).

The indices used to measure follicular antigen-capturing function in these studies were the mean follicular concentration of antigen and the mean follicular cross-sectional amount of antigen. As irradiation greatly reduced the size of the follicles, difficulty arises interpreting grain count data based on concentration. In the normal follicle the web of reticular cell processes is filled with lymphocytes (Mitchell and Abbot, 1965), and when these are destroyed by irradiation the total volume occupied by the radio-resistant (Jaroslow and Nossal, 1966b) antigen-capturing reticular cells is much reduced. Therefore measurements

of follicular antigen concentration following irradiation reflect not only the ability of each cell to capture antigen but also the density of antigen-capturing cells. For example, 1 day following irradiation the follicles had shrunk considerably, cross-sectional areas yielding values of one-third normal (Fig. 1). True functional damage to the follicular mechanism was minimal at that time so that the total numbers of grains in an average cross-section was only slightly reduced (Fig. 3), and the concentration of grains per unit was thus actually above normal values (Fig. 2). Considering the likelihood that equal numbers of reticular cells exist in a follicle post-irradiation the most meaningful index of cell function is found in the comparative ability of these cells collectively to trap antigen. Thus we believe the amount of and not the concentration of antigen present in an average follicle provides the best estimate of follicular cell function. It should be realized that added variation is introduced in determinations of follicular cross-section amounts of antigen, for size as well as concentration must be estimated. A random section through a lymph node may not include the largest follicles. Despite this objection results of determinations of mean follicular cross-sectional amount of antigen are consistent enough to provide a useful measure of follicular antigen capture (Fig. 3).

The clear cut and progressive reduction in the size of the follicles following irradiation may offer a partial explanation for the reduced ability of follicles to capture antigen. The collapsed follicle may well have a greatly reduced surface area of reticular processes thereby reducing possible areas of contact and capture of antigen. This could explain the finding that neither normal nor immune serum were found capable of increasing follicular antigen-capture completely to normal levels. However, the undoubted partial restorative effect of these agents shows that, as well as causing some anatomical damage, irradiation has indirect effects on the follicular trapping mechanism.

Irradiation might have reduced follicular antigen-capture by damaging the physiological processes that convey antigen from the footpad to the follicles of the regional nodes (Kahn, Kim, Curtis and Simons, 1960; Kahn, 1962). The evidence against such an effect can be summarized as follows: the gross distribution of polymerized flagellin at the site of injection and regional lymph nodes was unaltered at those time points following irradiation at which follicular uptake was most severely depressed; further the follicles in popliteal lymph nodes shielded during irradiation functioned normally even though the dose of whole body irradiation was nearly equivalent to that in non-shielded animals; the follicles failed to take up antigen in animals that had bone marrow or appendix shielded despite the fact that all of these animals survived irradiation in good health; regional injections of normal or immune serum improved follicular antigen capture but had no effect on the gross distribution of antigen; and intravenous infusions of large numbers of autogenous thoracic duct lymphocytes significantly improved follicular antigen-uptake in the thoracic duct depleted rat and also in the irradiated rat in the limited experiment conducted. It appears reasonable to conclude that the reduction in follicular antigen-capture following irradiation results from specific effects on lymphoid tissues.

As noted above, the finding that normal serum significantly improved follicular antigen-uptake at 10 days following irradiation provides the strongest argument in favour of the concept that opsonic factors are important in localizing antigen to follicles. However, it is possible that serum replenishes non-specific factors including vitamins, hormones or complement, and that a non-specific trophic effect of serum was of primary importance. However, foetal calf serum was an ineffective restorative agent in the irradiated rat. Despite the objections attendant on the use of heterologous serum, it is quite difficult to

conceive of a non-specific factor found in adequate concentration in 0·01 ml of immune serum and not in 0·25 ml of foetal calf serum. There was no consistent effect of either normal or immune serum on footpad retention or popliteal node uptake of antigen. The only consistent effect was to increase the uptake of antigen by follicles an average of five times which represents an altered *internal* distribution of antigen within the lymph node structure.

The substance in normal serum which acts as an opsonin for follicular antigen capture remains unknown. Of interest was the observed rate of decline in antigen-uptake by follicles at intervals following irradiation. Considerable variation was present, but a logarithmic decline is suggested. A reasonable estimate for the half life decline is 2·8 days. If the 'follicular opsonin' were an immunoglobulin this rate of decline in follicular function might represent its half-life in the follicles, which might be longer than that in serum in view of the finding of Ada *et al.* (1964) that autogenous γ-globulin was taken up actively by follicles. The rat antiflagellar IgG antibody has been found to have a serum half life of 5–6 days; the antiflagellar IgM antibody has a much shorter serum half life of 18 hours (Jaroslow, Grenot and Nossal, 1966). Thus the follicular opsonin may be a macroglobulin on the basis of the rate of decay. This interpretation is consistent with the findings of Reade, Turner and Jenkin (1965) who reported that the natural opsonic antibody directed against another species of *Salmonella* was IgM. The nature of the cell producing the follicular opsonin is unknown, but the small lymphocyte appears to be the best possibility. Plasma cells are not extensively destroyed by sublethal irradiation (Dixon and McConahey 1963; Taliaferro, Taliaferro and Jaroslow, 1964) or thoracic duct drainage, nor are reticular cells. Direct evidence implicating the small lymphocyte in the production of follicular opsonins is found in the improved follicular antigen-uptake following intravenous infusion of thoracic duct lymphocytes. The time intervals following lymphocyte infusions and test of antigen uptake were considerable, and it is possible that transformation of cell types occurred. Of note was the clear cut failure of any static lymphocyte preparation injected regionally to improve follicular antigen capture. The fact that lymphocyte extracts and regional inoculations of lymphocytes themselves are inactive suggests that if the small lymphocyte does secrete opsonic substances it does so in small amounts over a long period of time.

The failure of appendix-ileocaecal node shielded animals to show improvement of follicular antigen trapping following irradiation remains to be explained. It is possible that shielding the test nodes may have provided higher concentrations of lymph node products in this area than could have been achieved by shielding a distant lymphoid structure. However, the lymphoid system is highly dynamic (Jacobson, Simmons, Marks, Robson, Bethard, and Gaston 1960; Hall and Morris, 1964). If one lymphoid organ is shielded, active lymphocytes migrate to other areas rapidly (Sussdorf, 1960), and the concept of a high local production accounting for the difference observed is not entirely justified. The cells of the appendix and ileocaecal node may represent a large proportion of cells having functions different from those of peripheral lymph node lymphocytes (Archer, Sutherland and Good, 1963). Shielding of the test nodes provided these nodes with an intact follicular reticulum (Fig. 7c) which may have contributed further to better follicular antigen-uptake.

The functions of the follicular antigen trapping mechanism remain unclear. Whether depressed follicular uptake of antigen contributes to the lower antibody response of lymphopenic animals is open to question. These studies have utilized follicular antigen-uptake

largely as a sensitive indicator of levels of naturally-occurring opsonins. The tentative conclusion drawn from these studies is that thoracic duct or peripheral lymph node lymphocytes or their progency elaborate opsonic substances found in normal serum which enhance follicular antigen uptake. If these substances prove to be specific 'natural antibodies', the possibility exists that the small lymphocyte is committed to specific antibody production even in the unstimulated animal. Further work regarding the molecular nature and specificity of these serum factors is in progress to test this possibility.

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