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Antigens in Immunity

XVI. A LIGHT AND ELECTRON MICROSCOPE STUDY OF ANTIGEN LOCALIZATION IN THE RAT SPLEEN*

JUDITH MITCHELL AND A. ABBOT

The Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Melbourne, Victoria 3050, *Australia*

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Summary. This paper describes the ultrastructural location of labelled antigens and carbon in the spleens of rats from 4 minutes to 5 days after injection. Particular attention was focused on the sites of deposition 4 minutes after intra-arterial injection of microgram quantities of ^{125}I-labelled *Salmonella* flagellar antigens, crayfish haemocyanin and BSA, using colloidal carbon for comparison. The combination of radioautography with both light and electron microscopy showed the importance of antigen binding by lymphocytes in the marginal zone of the spleen. Macrophage sequestration of antigens was not prominent in the spleen, although it occurred in the liver with the flagellar antigens and haemocyanin.

In the spleen marginal zone, avid antigen-binding cells were found *in situ* 4 minutes after the injection of labelled haemocyanin. These appear to be the counterpart *in vivo* of antigen-binding lymphocytes prepared *in vitro*. Such cells also occurred infrequently after the injection of labelled polymerized flagellin, but were not found with either BSA or carbon.

The apparent movement of flagellar antigen from the marginal zone to the white pulp between 1 and 2 hours after injection was seen to involve lymphocyte-associated antigen. The follicular antigen localization occurring from 1 day onwards after injection was on the dendritic reticular cells of germinal centres, as has been described in lymph nodes after subcutaneous injection.

Carbon particles were rapidly sequestered in macrophages of the spleen and liver, although some particles were found between cells in the marginal zone for as long as 2 hours after injection. By 2 and 5 days, however, all the carbon was in phagocytes, even in the white pulp. Differences between the localization of antigens and carbon were clear, even in the ultrastructural sites of their location in tingible body macrophages of germinal centres.

The unexpected emphasis of lymphocyte association with labelled antigens in the spleen marginal zone has allowed a revision of the mechanism previously proposed for the movement of antigens within the microenvironments of the spleen.

INTRODUCTION

An earlier paper in this series has described the localization and the apparent movement in the rat spleen of intravenously injected antigens and carbon (Nossal, Austin,

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Pye and Mitchell, 1966). A point of interest in that study was the occurrence of heavily labelled cells in the marginal zone. These were assumed to be phagocytic cells, which posed problems in the interpretation of the apparent movement of antigen from the marginal zone to the white pulp of the spleen. With the recent description of antigen binding by lymphocytes *in vitro* (Naor and Sulitzeanu, 1967; Mandel, Byrt and Ada, 1969) it became important to examine ultrastructurally the sites of antigen retention in the spleen, particularly at short intervals after injection.

This paper describes the early lodgement sites of antigen in the rat spleen after the injection of small quantities of *Salmonella* flagella antigens, crayfish haemocyanin and bovine serum albumin labelled with ^{125}I. It compares the distribution of these antigens with the essentially non-antigenic carbon particles of india ink. Intra-arterial injection via the aorta and splenic artery was used to achieve maximum localization in the spleen for the relatively insensitive procedure of electron microscopic radioautography.

MATERIALS AND METHODS

Animals

Adult rats of the randomly bred Wistar or inbred DA strains were used at 8–19 weeks of age. Since no differences emerged during the study, the results from animals of both strains have been pooled.

Materials for Injection

Polymerized flagellin from *Salmonella adelaide* was prepared by the method of Ada, Nossal, Pye and Abbot (1964a). Whole flagella from the same organism were also used. Haemocyanin from the crayfish *Jasus laldandii* was kindly provided by Professor G. L. Ada. Bovine serum albumin (crystalline) was purchased from Armour Chemicals and was dissolved in distilled water for use. India ink (Pelikan Waterproof Ink) was centrifuged at approximately 1500 ***g*** for 5 minutes and the supernatent diluted 1:2 or 1:10 in phosphate buffered saline (pH 7·3) for injection.

Isotope and Iodination

Carrier-free ^{125}I was purchased from the Radiochemical Centre, Amersham, U.K. Proteins were iodinated by the chloramine-T oxidation method described in detail by Ada, Nossal and Pye (1964b). Ten-fold lower concentrations of chloramine-T and sodium metabisulphite were used for the iodination of haemocyanin. The amount of radioactivity and protein in each preparation is recorded in Table 1.

Intra-arterial injection

Injections were made into the abdominal aorta by a technique developed in collaboration with Mr Jeffrey Fyffe. Under Nembutal anaesthesia, a 30-gauge steel needle bent to an angle of 70° was inserted into the aorta superiorly to its coeliac branch. The gastric and hepatic branches of the coeliac artery were clamped and the aorta occluded with a small clip between the junctions of the coeliac and mesenteric arteries just before inserting the needle. A volume of about 0·2 ml was injected over a period of 15 seconds and then the aortic clamp was removed. When animals were to be kept for longer than 4 minutes after injection, the needle was withdrawn slowly, 2 minutes after injection, and the hepatic

artery clamp removed. The abdominal wall was then sutured and the animal allowed to recover from the anaesthetic.

Immunizations

Rats were either actively immunized or had been injected intravenously with 0·9 ml of pooled antiserum from the same strain 4 hours before intra-arterial injection. The antiserum had a titre of 6400 by immobilization titration (Ada *et al.*, 1964a) and at the time of spleen removal the serum titre of recipient rats was about 400.

Preparation of tissues for histology

Tissue for light microscopy radioautography was fixed in 10 per cent formalin buffered to pH 7·3, and for electron microscopy in 2·5 per cent glutaraldehyde in 0·1 M phosphate buffer with postfixation in 1 per cent osmium tetroxide in veronal acetate buffer (pH 7·3). In some cases, material for electron microscopy had remained in formalin for 1 week before fixation in glutaraldehyde and osmium.

Organs to be examined 4 minutes after injection were removed under the Nembutal anaesthesia used for injection, whilst those taken at later times were from rats anaesthetized with ether. Pieces of tissue 2 mm thick were cut for light and electron microscopy and were processed by the method described in detail by Nossal, Abbot and Mitchell (1968a).

Paraffin embedded tissue was sectioned at 5 μ, dipped in Kodak NTB-2 emulsion and exposed for periods ranging from 4 hours to 60 days. After development in Kodak D19b the sections were stained with methyl green–pyronin.

Araldite embedded tissue was assayed by counting in a sodium iodide well crystal. The three blocks with the highest counts were selected and a survey section of 1–2 μ cut. This section was processed for radioautography with NTB-2, exposed for 1–5 days, developed and stained with 1 per cent toluidine blue. A labelled area was selected and the block trimmed to about 0·7 mm^2. A ribbon of silver-coloured sections was cut, terminating in a 1 μ section for light microscopy. Ultrathin sections were prepared for radioautography by the method of Salpeter and Bachmann (1964) with modifications (Nossal *et al.*, 1968a). The sections coated with Kodak NTE emulsion were exposed for 1–8 months, developed in Dektol and mounted on either annular copper discs or mesh supports before viewing in a Philips EM300 microscope.

RESULTS

LIGHT MICROSCOPE LOCATION OF LABELLED ANTIGENS AND CARBON

A. *Distribution in the spleens of normal rats 4 minutes after injection*

(i) Salmonella *flagellar antigens*. Two rats were injected with labelled polymerized flagellin (Table 1). Radioautographs of the spleens removed after 4 minutes showed label concentrated in the marginal zone, with less in the red pulp and virtually none in the white pulp.

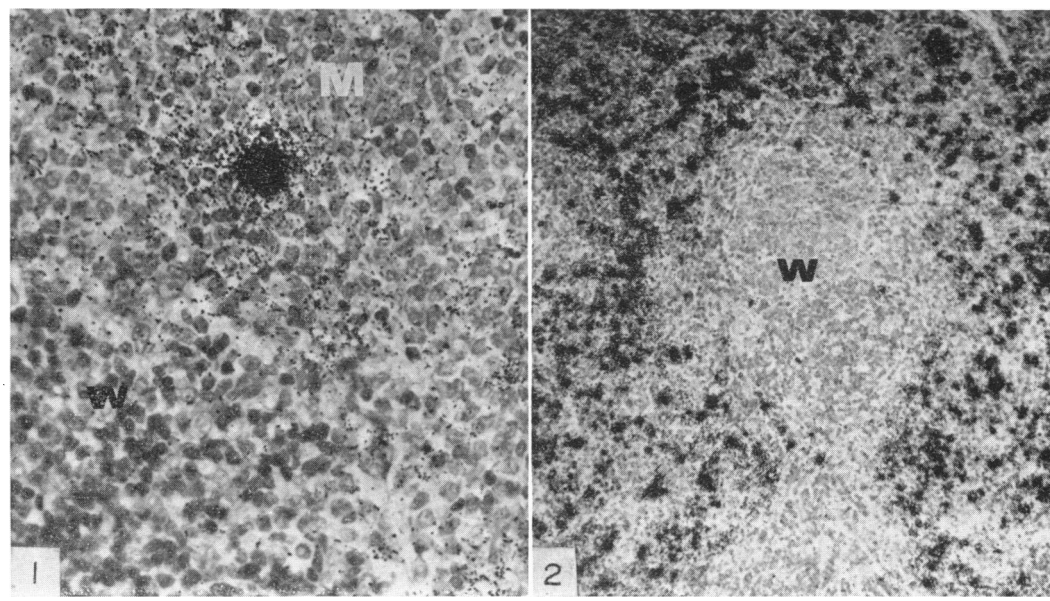
Serial radioautographs exposed for different lengths of time yielded apparently different pictures of the localization of label. On sections exposed for 1 day there were isolated heavy clumps of grains (Fig. 1) which were some 5 times more common in the marginal zone than the red pulp. Other label was present, scattered in an irregular pattern

TABLE 1

REPRESENTATIVE DATA FROM RADIOACTIVITY COUNTING OF ^{125}I-ANTIGENS INJECTED INTRA-ARTERIALLY INTO RATS WHICH WERE KILLED 4 MINUTES LATER

Antigen	Amount of radioactivity injected (cps × 10^{-6})	Protein (μg)	Specific Activity (counts/sec/mg)			
			Blood	Spleen	Liver	Mesenteric node
Polymerized flagellin (Pol)	1·7	3·3	214	131	59	5
	6	4·6	294	160	140	14
Whole flagella	2·6	4·7	296	338	330	17
Haemocyanin	9·2	1	105	293	468	8
BSA	17	31	796	115	136	73
Pol plus antiserum	1·4	2·6	45	166	67	1
Flagella plus a/serum	2·2	4·1	28	66	11	3
Pol into * immune rat	8·8	3·2	296	388	346	2

* Rat killed 1 minute after injection.



FIGS 1 and 2. [^{125}I]polymerized flagellin in the spleen 4 minutes after injection. M = marginal zone, w = white pulp. (1) A heavily labelled cell in the marginal zone. Exposure, 1 day. Magnification, × 325. (2) Overall pattern of label distribution. A few labelled cells appear in the white pulp. Exposure, 30 days. Magnification, × 130.

throughout the marginal zone and red pulp, but the white pulp was unlabelled. Sections exposed for 7 days showed an increased number of clumps of grains and increased intensity of the scattered label. After long (4 weeks) exposure, the marginal zone was labelled throughout with dense patches of grains. The rare heavy clumps, apparent after 1 day's exposure, could no longer be distinguished. Another feature of the long radioautographic exposure was the appearance of labelled cells in the white pulp where they were sometimes inside arterioles, but more often appeared to be in the periarteriolar lymphocyte sheath (Fig. 2).

When serial sections of the spleen were compared on radioautographs exposed for 1 day, the rare heavy label was seldom present in the same location on consecutive 5 μ sections. With 1 μ sections of araldite-embedded material, such label was sometimes seen on two or three consecutive sections, but when the grains were dissolved using Farmer's solution (Humphrey and Keller, 1970) the label always seemed to have originated from a point between cells, rather than from over or around one particular cell. Further identification of the origin of such intense label required the increased resolution of electron microscopy and is described later.

Whole flagella from *Salmonella adelaide* appeared to be distributed like polymerized flagellin 4 minutes after intra-arterial injection.

(ii) *Haemocyanin.* Four minutes after the injection of labelled haemocyanin, radioautographs of the spleen exposed for only 4 hours showed occasional heavy clumps of grains in the marginal zone and red pulp. These clumps were similar in their distribution to those found with the flagellar antigens. Light irregular label was also present in these zones and the white pulp was unlabelled. Longer exposure (1–7 days) brought up a pattern of heavy diffuse label somewhat different from that seen with the flagellar antigens; the white pulp was labelled only where an arteriole was present in cross section, but the marginal zone and red pulp exhibited uneven lines, curves and patches of confluent label (Fig. 3). These streaks of label did not appear to be composed of many over-exposed

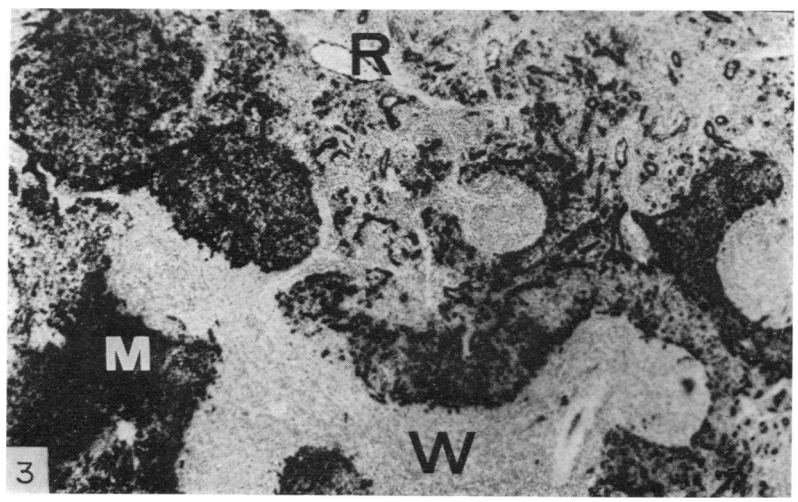


FIG. 3. [^{125}I]haemocyanin in the spleen 4 minutes after injection. Label is concentrated in the marginal zone (M). R = red pulp, w = white pulp. Exposure, 7 days. Magnification, $\times$ 60.

single cells, but rather appeared to have originated from local concentration of the label in sinuses or around tissue spaces.

(iii) *Bovine serum albumin (BSA).* The spleen of one rat injected with 2 μg labelled BSA did not retain sufficient radioactivity for radioautography, but when 30 μg BSA containing 900 μCi ^{125}I was injected, satisfactory results were obtained. In contrast to the other antigens, BSA was most concentrated in the afferent and efferent vessels of the spleen 4 minutes after injection, and the label was diffusely distributed in each of the splenic

compartments. No heavy clumps of grains were seen with this antigen, although there were occasional cells somewhat more heavily labelled than the surrounding region. After long (60 days) exposure the radioautographs showed streaks of confluent grains in the marginal zone, red pulp and white pulp arterioles, similar to the long exposures of spleen sections from the haemocyanin-injected rats. Within the white pulp, label was diffusely distributed but was less concentrated than in the marginal zone or red pulp.

(iv) *Carbon.* When india ink was injected intra-arterially in a small amount (0·2 ml 1:10 dilution) carbon particles were found diffusely distributed in the marginal zone of the spleen 1 minute later. Very little could be seen in the red pulp by light microscopy. Four minutes after an intravenous injection of 0·2 ml of 1:2 dilution, the particles were again mainly in the marginal zone with some isolated patches in the red pulp. Within the marginal zone the carbon particles appeared to be intercellular but there was no evidence of heavy collections around one particular cell. No carbon was seen in the white pulp.

B. *Distribution of flagellar antigens in the spleens of immunized rats*

Rats which had either been actively immunized or received antiserum before the injection of labelled antigen showed an increased uptake of radioactivity in the spleen. This was shown by comparison of the specific activities of spleen and blood at the time of killing (Table 1). The radioautographs of their spleens showed a difference from normal in the pattern of label distribution. One day exposures of light microscope sections showed that much more of the antigen had been confined to the marginal zone in both actively and passively immunized rats. Even with 1 day radioautographic exposure, the label was composed of many heavy clumps of grains in the marginal zone. There was light scattered label in the red pulp and the white pulp was unlabelled.

C. *Distribution of antigens and carbon in the liver, 4 minutes after injection*

Heavy accumulations of label were seen over Kupffer cells of the liver after the injection of polymerized flagellin, whole flagella or haemocyanin. This label was present only in sinusoids on radioautographs exposed for 1 day and was comparable in intensity to the isolated heavily labelled cells in the spleen (Fig. 1). On longer exposure, light scattered label was also seen over parenchymal cells.

After the injection of BSA, on the other hand, the liver showed diffusely distributed label which was most concentrated in the portal vein regions and decreased outwards in a radial pattern. The Kupffer cells were not specifically labelled.

Carbon particles were retained in the liver macrophages in the same pattern as label after the injection of the flagellar antigens and haemocyanin.

D. *The localization of polymerized flagellin at later times*

During the first day after injection, an apparent movement of antigen occurs within the spleen. Label appears to move from the marginal zone, across the marginal sinus into the outer border of the white pulp in the first 2 hours. By 24 hours, the label is concentrated in the germinal centre caps of the white pulp where it may remain for weeks. The pattern of apparent antigen movement, which has been described in detail after intravenous injection (Nossal *et al.*, 1966) also occurred after the intra-arterial injection used in the present experiments. Attention to short exposure times, however, showed that on radioautographs of the spleen 1 and 2 hours after injection, a few individual heavily

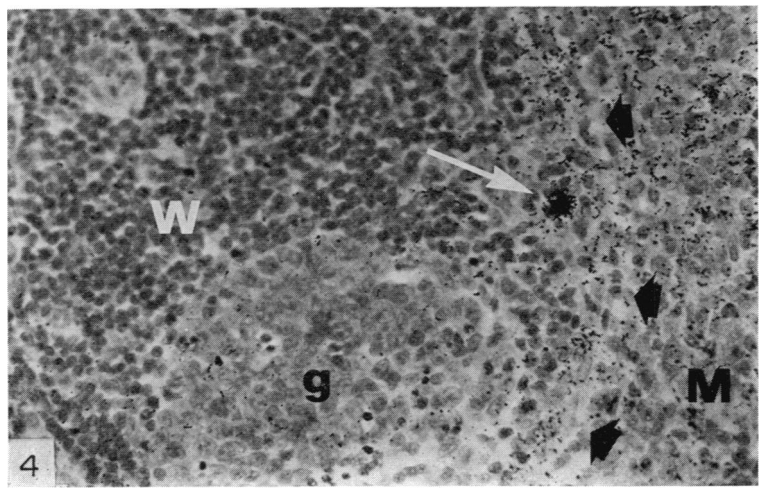


FIG. 4. [^{125}I]polymerized flagellin in the spleen 2 hours after injection. A labelled cell (white arrow) lies in the white pulp (w) which contains a germinal centre (g). Black arrows indicate marginal sinus. M = marginal zone. Exposure, 1 day. Magnification, ×280.

labelled cells occurred in the region of the marginal sinus. Some of these (Fig. 4) were inside the white pulp near the follicular cap region of germinal centres. No such labelled cells were found in rats killed 1 or 2 days after injection.

An interesting contrast emerged between the degree of labelling in the liver and spleen when sections were compared after the same exposure periods. The Kupffer cells of the liver were more heavily labelled 1 and 2 hours after injection than were most of the cells in the spleen red pulp; yet with intra-arterial injection all the antigen reaching the liver must first have passed through the spleen.

ELECTRON MICROSCOPE LOCATION OF LABELLED ANTIGENS AND CARBON IN THE SPLEEN

A. *The distribution of label in normal rats 4 minutes after injection*

(i) Salmonella *flagellar antigens*. Although label was always found in the ultrathin sections in the areas indicated by the matching light microscope radioautograph, the identification of the heavily labelled cells (Fig. 1) proved to be difficult. Only once did we positively identify heavy marginal zone label in both the light and electron microscopes. The area was labelled in four out of five serial ultrathin sections (Fig. 5) and contained a cell with a nucleus of irregular outline, peripheral condensation of chromatin and a prominent nucleolus. Its cytoplasm had few organelles but many ribosomes and the outer membrane, with which the label seemed to be associated, had many complex infoldings. The cell was typical of the many which were difficult to classify but it appeared to be 'lymphocyte-like'.

In the search for heavily labelled cells in the marginal zone, many isolated clumps of grains were found extracellularly, associated with the cytoplasmic membranes of various types of cells. Although lymphocytes were frequently the only cells present near a clump of grains, label was also present between lymphocytes and macrophages and sometimes overlying dense cytoplasmic material which appeared to be part of the extracellular reticulum. Label was seldom found in macrophages in the marginal zone.

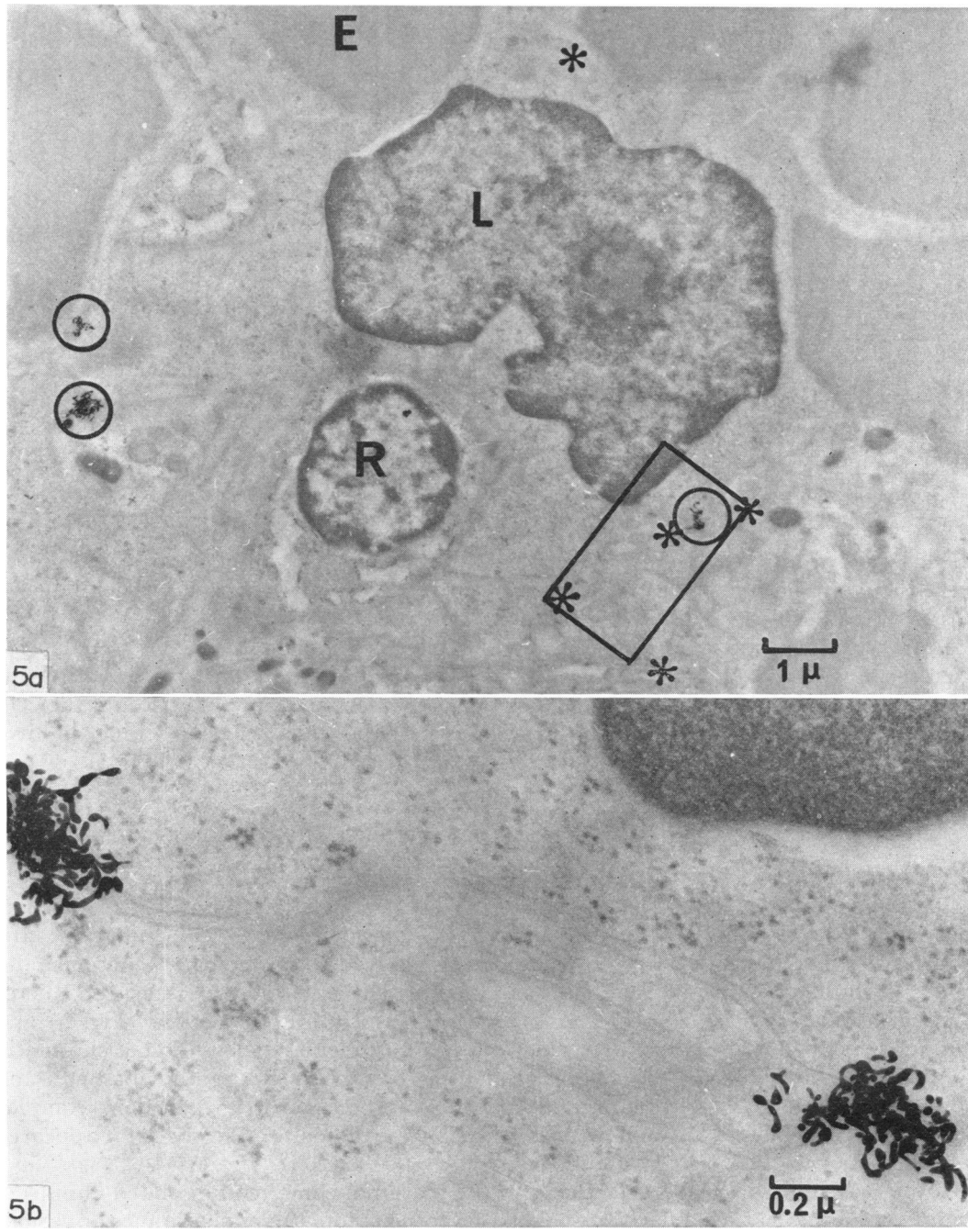
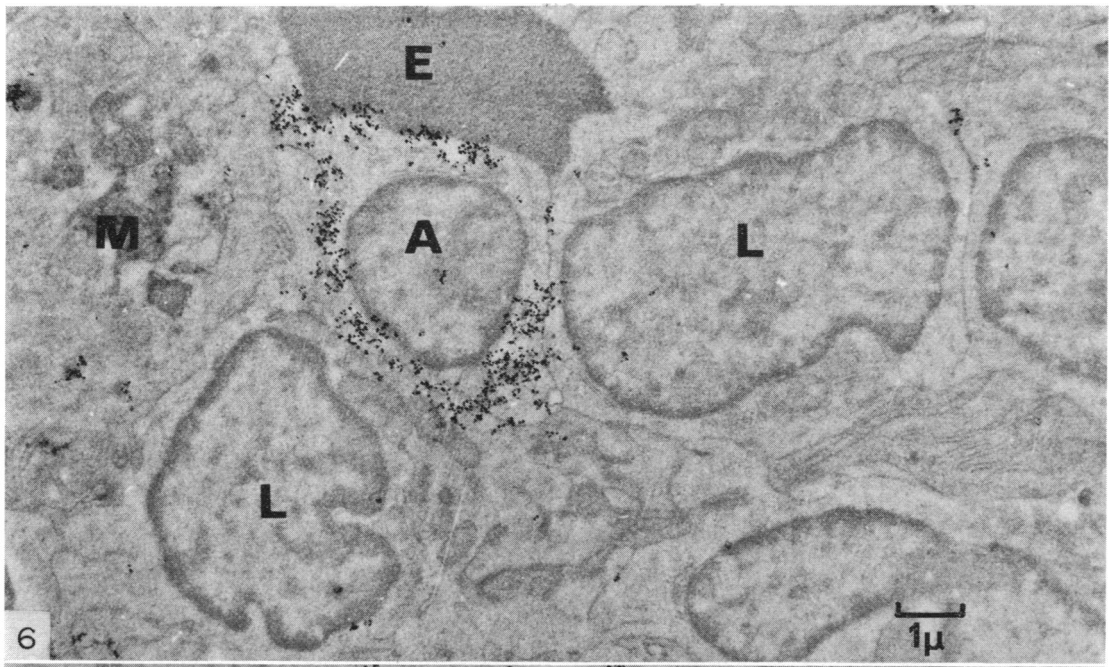


FIG. 5. [^{125}I]polymerized flagellin in the spleen 4 minutes after injection. Area matching heavily labelled cell in light microscope (see text). Exposure, 3 weeks. (5a) (× 10,200) Field contains a lymphocyte (L), erythrocytes (E) and a reticular cell (R). The grains are circled and asterisks indicate areas found labelled on three other serial sections. The oblong shows the area of: (5b) (× 51,700) High magnification to show the membrane association of label (from the next section in series with 5a).



FIGS 6 and 7. [^{125}I]haemocyanin in the marginal zone of the spleen 4 minutes after injection. Unstained sections. Exposure 18 weeks. (6) Avid antigen-binding lymphocyte (A) amongst other lymphocytes (L), an erythrocyte (E) and a macrophage (M). Magnification, ×8300. (7) A more lightly labelled lymphocyte showing grains in patches in association with membranes. Magnification, × 14,900.

In red pulp there was good correlation between the labelled areas of the light and electron microscope radioautographs. Some grains were in cytoplasmic vacuoles of cordal macrophages containing endogenous ferritin and abundant lysosomes. Other label in this region was extracellular on the membranes of lymphocytes and other cells; none was found free within vessels.

(ii) *Haemocyanin.* A large amount of label was found in the spleen when this antigen was used. Six striking examples of label specifically associated with an individual cell were found in the marginal zone (Fig. 6). These cells most often showed peripheral label some of which was intracellular, not apparently associated with infoldings of the plasma membrane. The labelled cells were of similar morphology; the nucleus was lymphoid and the cytoplasm had few organelles and many long infoldings of the cytoplasmic membrane (Fig. 7). Less concentrated label was found associated with other lymphoid cells. Some typical macrophages showed labelled vacuoles (Fig. 6) and extracellular label was found in areas of complex cytoplasmic projections. The extraordinary intimacy of cell interdigitation frequently seen in association with label is shown in Fig. 8.

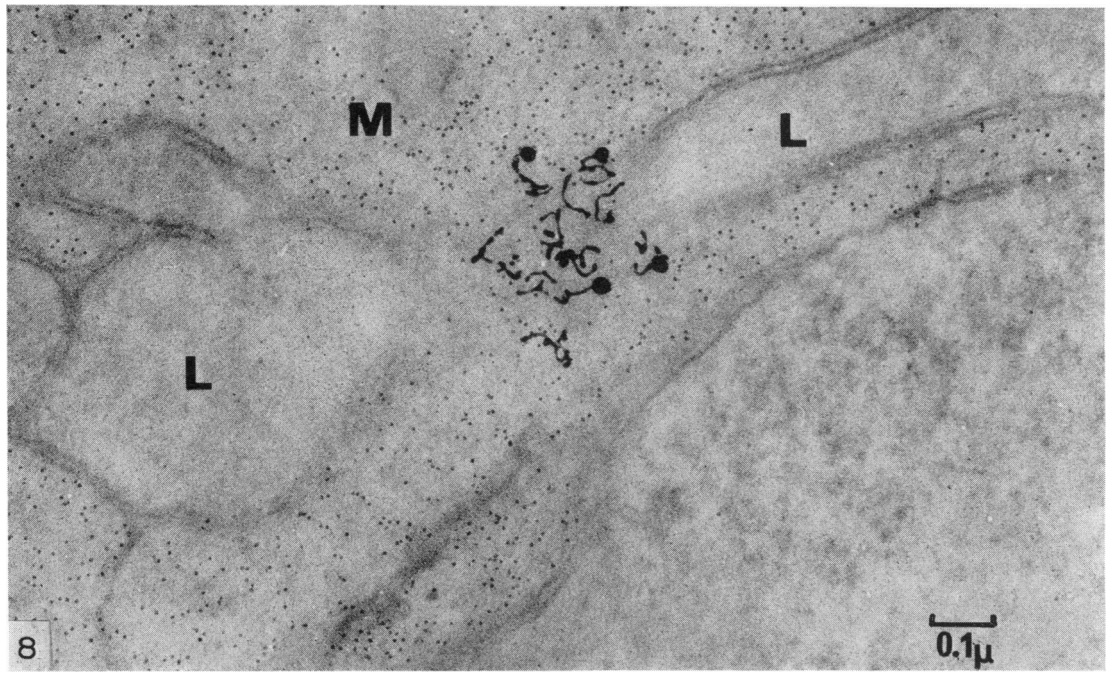


FIG. 8. [^{125}I]haemocyanin in the spleen 4 minutes after injection. Silver grains associated with tangentially cut membranes between a macrophage (M) containing endogenous ferritin and a lymphocyte (L). Unstained section. Exposure, 15 weeks. Magnification, × 83,700.

(iii) *Bovine serum albumin (BSA).* Despite the absence of heavily labelled cells with this antigen, clumps of grains were found in the electron microscope preparations of marginal zone and red pulp areas of the spleen. This label was again associated with the outer membranes of lymphocytes although no more than one patch was seen associated with one particular cell. Most label was seen in the marginal sinus region. Other clumps of

grains were found in the red pulp between cells of different morphology, and some seemed to be associated with the outer membranes of platelets.

(iv) *Carbon.* Carbon particles were found both within and between cells in the marginal zone 4 minutes after intravenous injection. There was a superficial similarity between the intercellular location of labelled antigen and some of the carbon in this region. The carbon, however, did not seem to be clustered around any one particular cell and often seemed to be associated with the dense extracellular reticulum (Fig. 15). Serial sections showed that the carbon particles were in the same location over several sections, which is in contrast to the findings with the labelled antigens. Carbon was present on the outer membranes and inside vacuoles of macrophages both in the marginal zone and red pulp as has been extensively described by Burke and Simon (1970).

B. *The distribution of polymerized flagellin in immunized rats*

In passively immunized rats after 4 minutes, label in the marginal zone was most commonly associated with the outer membranes of 'lymphocyte–like' cells. In the red pulp label was found in macrophage vacuoles as well as extracellularly in ill-defined areas. There was no qualitative difference apparent between the passively immunized and normal rats in the location of label.

Heavily labelled radioautographs were obtained from the spleen of an actively immunized rat 1 minute after intra-arterial injection. Grains in clumps were predominantly associated with the plasma membranes of 'lymphocyte-like' cells in the marginal zone, although some may have been intracellular (Fig. 9). No cells surrounded by label were

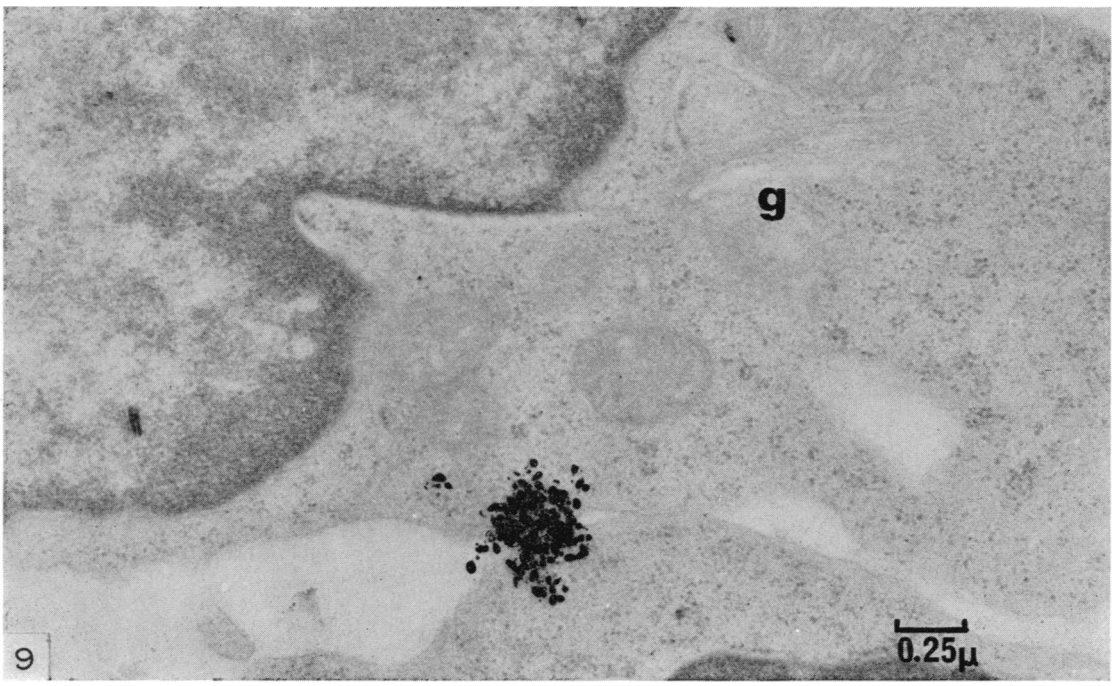


FIG. 9. [^{125}I]polymerized flagellin in the spleen of a pre-immunized rat, 1 minute after injection. Grains between two lymphocytes in the marginal zone. g = Golgi zone. Exposure, 30 weeks. Magnification, × 35,000.

found, but in areas matching heavy label in the light microscope, cells with two or three patches of grains on their surface were found in serial ultrathin sections. Such cells were lymphoid, often showing active cytoplasmic processes, a prominent Golgi zone (Fig. 9), a centriole, a few rounded mitochondria and an abundance of free ribosomes. Occasionally channels of endoplasmic reticulum running deep into the cytoplasm had label associated with them.

A difference between the normal and pre-immunized rats was apparent in the red pulp. In the immunized animal, label was frequently associated with platelets which were often clumped (Fig. 10). Label which appeared to be inside platelets was found, on

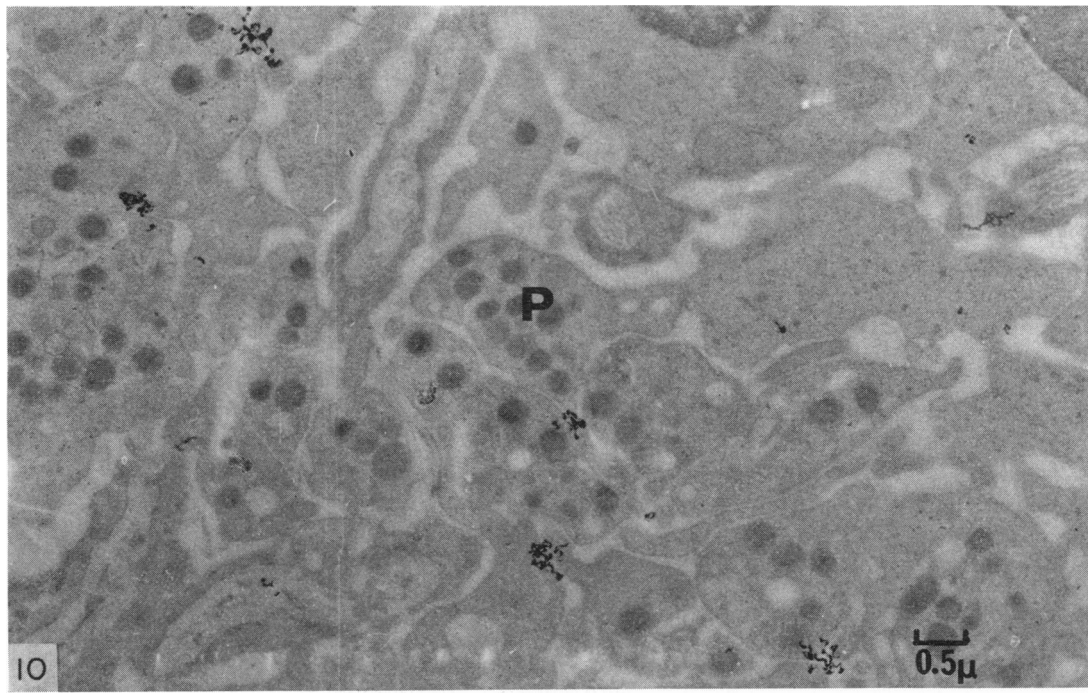
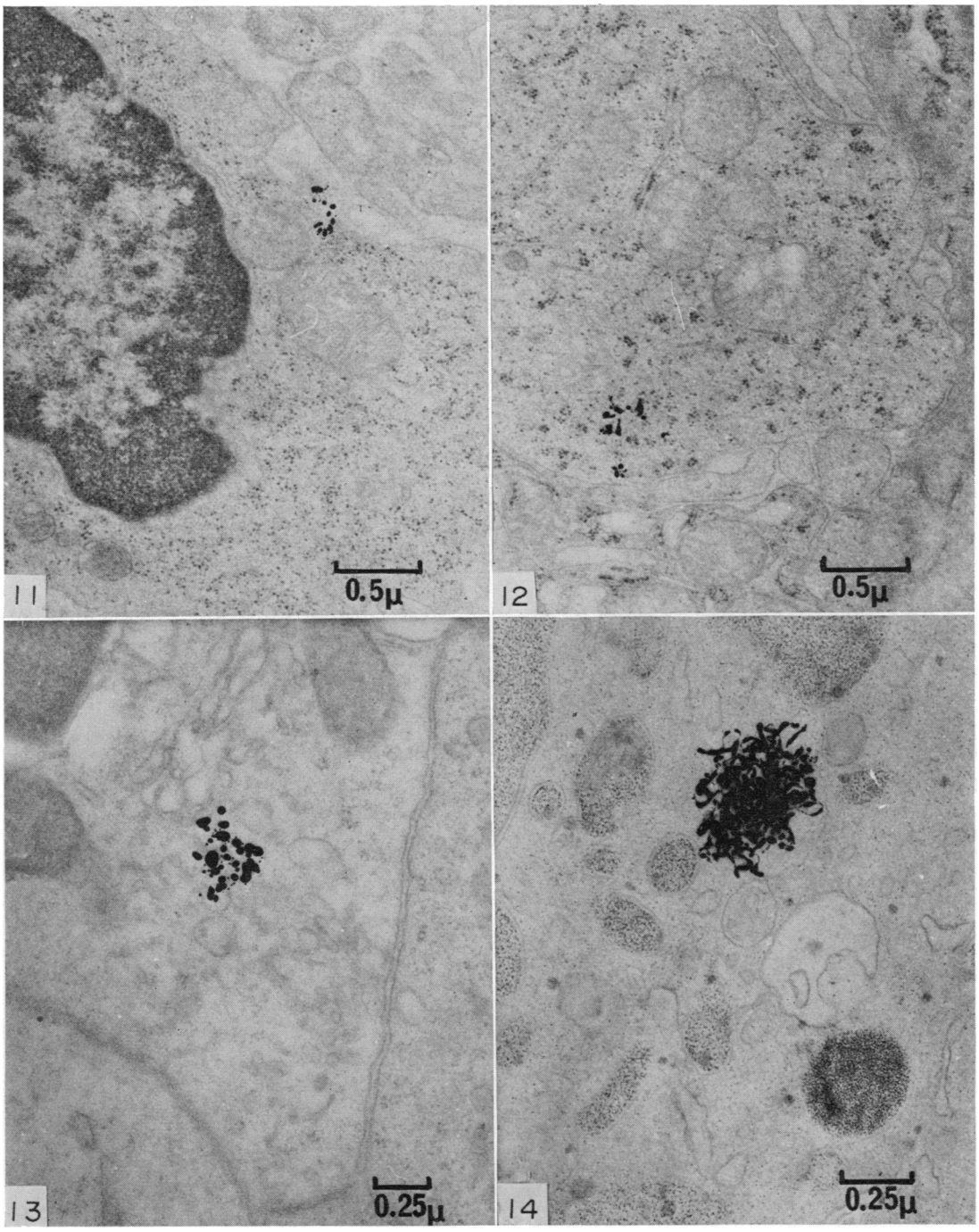


FIG. 10. [^{125}I]polymerized flagellin in the spleen of a pre-immunized rat 1 minute after injection. An example of platelets (P) in the red pulp, aggregated and with label associated. Exposure, 20 weeks. Magnification, × 14,100.

serial section, to be surface associated on a plasmalemmal invagination. Other label in the red pulp was between cells of different types. Cell interdigitation was a feature often seen underlying clumps of grains, and frequently the presence of endogenous ferritin was the only clue to the presence of macrophage cytoplasm in an area of lymphocytes.

C. *Nuclear label*

With the flagellar antigens and haemocyanin, small numbers of grains were sometimes found overlying the nuclei of cells which exhibited cytoplasmic labelling. This could never be found on serial sections.



FIGS 11 to 14. [^{125}I]polymerized flagellin in the spleen. Exposure, 16 weeks. (11) Membrane-associated label in the marginal zone 1 hour after injection. (12) Intracellular label in a lymphocyte of the marginal zone 1 hour after injection. (13) Intracellular label in a lymphocyte in the white pulp near the marginal sinus, 2 hours after injection. (14) Intracellular label in a macrophage of the red pulp.

D. *The localization of polymerized flagellin and carbon at later times*

(i) 1–2 *hours*. Membrane-associated extracellular patches of grains in the marginal zone was the most common location of label 1 and 2 hours after intra-arterial injection of polymerized flagellin. Despite long (4 months) exposure, the clumps of grains were small and most seemed to be on lymphocytes containing abundant ribosomes (Fig. 11). Occasionally the label was intracellular (Fig. 12) in lymphocytes and sometimes in association with closely apposing processes of lymphoid cells with the dense cytoplasmic material of the marginal zone, but it was seldom found in macrophages.

By this time some antigen had penetrated the outer borders of the white pulp. Label was hard to find in this region, but several cells subjacent to the marginal sinus contained significant numbers of grains either on their outer membrane or within their cytoplasm (Fig. 13). No label was seen associated with the dendritic follicular cells of germinal centres at this time.

In the red pulp, membrane bounded, ingested label was found in cord macrophages (Fig. 14) and in other phagocytic cells. There were still, however, grains associated with membranes of mononuclear free cells in this region.

Carbon particles were mostly inside vacoules of macrophages in both the marginal zone and red pulp 1 hour after intravenous injection. Nevertheless, some was still present intercellularly in the marginal zone (Fig. 15). In contrast to polymerized flagellin, carbon was not found in the peri-follicular regions of the white pulp.

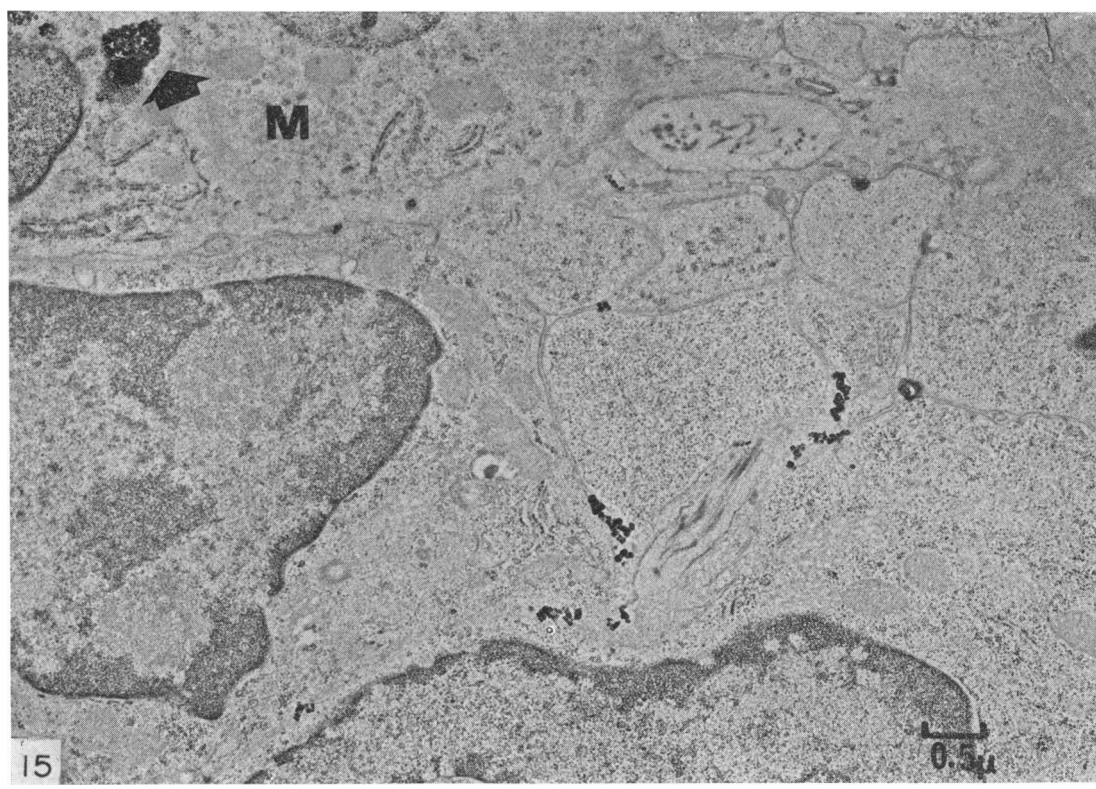


FIG. 15. Carbon particles in the marginal zone 2 hours after injection. While most are intracellular (arrow) in macrophages (M) some appear to remain extracellular. Magnification, × 14,400.

(ii) 4 *hours*. Radioautographs of antigen in the spleen after a foot pad injection (Nossal *et al.*, 1966) suggested that the label was associated with individual cells. For electron microscopy, one rat was injected into the hind foot pads with a total of 6 μg polymerized flagellin and the spleen removed 4 hours later. No heavily labelled cells were seen in the marginal zone where most of the label was found. Membrane association with lymphocytes was the main finding in this zone, with intracellular labelling of macrophages in the red pulp.

(iii) 2 *days*. From 1 day onwards after the injection of polymerized flagellin by the subcutaneous route, lymph node follicles show retention of antigen on the dendritic reticular cells (Nossal *et al.*, 1968a). In the present experiments, the same ultrastructural location of label was seen 2 days after intravenous injection of 6 μg polymerized flagellin into normal or preimmunized rats. Small numbers of grains were present over membranes of dendritic follicular cells and sometimes overlying their intracellular membranes. Occasionally, in the preimmunized rat, label was found inside a tingible body macrophage scattered throughout an inclusion formed by a degenerating cell. There was no evidence of phagocytosis of labelled antigen alone by tingible body macrophages.

Two and 5 days after intravenous injection of india ink, all the carbon seen by electron microscopy was intracellular. In the spleen white pulp, carbon was in vacuoles of macrophages in the periarteriolar lymphocyte sheath. Transitory follicular localization of carbon has been described previously (Nossal *et al.*, 1966) but none was found by electron microscopy in these studies. The only carbon seen in germinal centres was inside tingible body macrophages. The appearance of the vacuoles containing carbon was the same in macrophages of both the germinal centres and the periarteriolar sheath. These vacuoles varied in size but in the tingible body macrophages, carbon was seldom present in the same vacuole as a recently engulfed recognizable lymphocyte.

DISCUSSION

With the increased resolution of radioautography in the electron microscope we have answered some of the questions raised in our previous light microscope study of antigens in the rat spleen (Nossal *et al.*, 1966). A most interesting finding was the ultrastructural location of antigen in association with lymphocytes in the marginal zone, suggesting that this area may be a primary site for antigen–lymphocyte interaction.

The marginal zone contains macrophages which phagocytose carbon (Burke and Simon, 1970) and it has been assumed previously that these cells are also responsible for antigen retention as, indeed, are the macrophages of the lymph node medulla (Nossal *et al.*, 1968a). The present work, however, shows that it is the lymphocytes of the spleen marginal zone that are responsible for much of the antigen retention, even 2 hours after injection. Macrophage association is not prominent with label found 4 minutes after the injection of labelled flagellar proteins, crayfish haemocyanin or BSA. The fact that 4 minutes is sufficient time for phagocytosis to occur was evident from the accumulation of flagellar proteins and haemocyanin in vacuoles in the Kupffer cells of the liver. Furthermore, carbon was found intracellularly in macrophages of both the spleen and liver at this time.

Avid antigen binding cells, that is, lymphocytes far more heavily labelled than their neighbours, were difficult to demonstrate by electron microscopy with the flagellar antigens. This was due to their infrequent occurrence and the small amount of tissue

which can be cut in one section for electron microscopy. Haemocyanin, on the other hand, yielded six avid antigen-binding cells in three electron microscope sections. These were lymphocytes which appear (Fig. 6) to be the counterpart *in vivo* of the avid antigen-binding cells prepared *in vitro* and demonstrated by Mandel, Byrt and Ada (1969). No such cells were found with BSA.

The distribution of the grains found associated with lymphocytes after the injection of all three antigens suggested that antigen was attached to the outer cell membrane in discrete patches. Carbon particles of india ink, on the other hand, tended to lie together in streams. The extracellular particles could be found in the same locations through five serial sections and the most likely explanation for this seems to be trapping in the marginal sinus by sluggish circulation and the close approximation of the cells in this zone. Furthermore, extracellular carbon particles were not located in patches or continuously around any one particular cell. Antigen patches were not always located in two serial sections and the same patch was never located in more than two sections. This patchy nature of antigen localization corresponds to the distribution of both antigen and antibody traced by various techniques in other studies (Mandel, Byrt and Ada, 1969; Jones, Marcuson and Roitt, 1970; Aoki, Hammerling, de Harven, Boyse and Old, 1969).

In locating labelled flagellar antigens in either passively or actively immunized rats we found no evidence of increased macrophage uptake. Although radioautography in the electron microscope cannot be regarded as a quantitative technique for antigen localization, our findings suggested that antibody has the effect of increasing the number of lymphocytes capable of binding antigen on their surface. It now seems more likely that antibody increases the number of cells involved in antigen trapping rather than actually hastening the events of antigen sequestration, as was proposed previously (Nossal *et al.*, 1966).

In the light microscope studies, the use of a range of radioautographic exposures proved to be important. In fact, the apparent pattern of antigen localization was altered by long exposure. The emphasis given to the few avid antigen-binding cells by short exposure was lost by the long exposure needed to show the overall pattern of antigen localization. On the other hand, short exposure did not reveal labelled cells inside the white pulp 4 minutes after antigen injection.

The ultrastructure of labelled cells in the white pulp 4 minutes after the injection of the flagellar antigens (Fig. 2) could not be determined in these experiments. It seems likely from their morphology in the light microscope that they were lymphocytes rather than macrophages. In explaining the presence of such cells we are hindered by the lack of information about the circulation of fluid in the microenvironment of the white pulp. Very little blood enters this area, although red cells are occasionally seen amongst the lymphocytes. Our work gave no suggestion of antigen leakage from the central arteriole. It is possible that antigen is carried by plasma circulating into the white pulp from the marginal zone, when antigen binding cells in the periarteriolar lymphocyte sheath would have the opportunity to become labelled. On the other hand, antigen binding cells labelled in the marginal zone may have migrated into the white pulp even within 4 minutes. Information from the injection of antigen-binding lymphocytes may show whether the latter is feasible; such experiments are in progress.

Within an hour after the injection of polymerized flagellin, antigen appears to have moved from the marginal zone to the outer regions of the white pulp. The demonstration of antigen binding by lymphocytes in the marginal zone allows a more attractive mechanism for this movement than was previously discussed (Nossal *et al.*, 1966). In the normal

animal, lymphocytes can be seen in passage across the marginal sinus. We searched for the carriage of antigen by such cells and found a few labelled lymphocytes just inside the white pulp both 1 and 2 hours after injection. The label of these cells was found either associated with the outer membrane or, indeed, intracellularly (Figs 12 and 13).

The ultrastructural location of follicular label in the spleen one day and longer after the injection of polymerized flagellin was the same as that found in lymph nodes (Nossal *et al.*, 1968b). Antigen appears to be associated with the dendritic reticular cells and label is occasionally seen throughout an inclusion formed in a tingible body macrophage by the engulfment of a near mitotic lymphocyte (Odartchenko, Lewerenz, Sordat, Roos and Cottier, 1967). As was found in the lymph node germinal centres, tingible body macrophages did not appear to engulf labelled antigen, except when it was associated with a lymphocyte. Further work is in progress to see whether these macrophages engulf labelled cells in the absence of follicular antigen deposits on dendritic reticular cells. The present study has provided no definite link between the labelled lymphocytes in the perifollicular white pulp areas at 2 hours and the ultimate dendritic reticular cell localization of antigen found from 1 day after injection.

The localization of carbon in the white pulp 2 and 5 days after the injection of india ink was in contrast to the localization of polymerized flagellin. Carbon was present in macrophages throughout the white pulp in vacuoles of various sizes. Although carbon was present in the tingible body macrophages of germinal centres, it was generally not in association with a recently engulfed lymphocyte. Thus there appeared to be a difference between the location of carbon and antigen even when they were found in the same type of cell.

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