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Antigen in Tissues

III. THE SEPARATION OF ANTIGEN-CONTAINING COMPONENTS FROM LYMPHOID TISSUES

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Summary. Particulate and soluble antigens were labelled with ^{125}I, injected into rats and the lymphoid organs examined. As determined by autoradiography of tissue sections, one antigen used localized exclusively in vacuoles of medullary macrophages of the lymph nodes and others on the surfaces of reticular cells in the lymphoid follicles of nodes or the white pulp of spleen. The remaining antigens studied localized in both medulla and lymphoid follicles of nodes.

Tissues containing antigen were homogenized in a sucrose medium and most radioactivity was recovered in a large granule fraction. This fraction was submitted to equilibrium centrifugation. The preparations were not resolved in gradients of sucrose or dextran but in gradients of Urografin the preparations were resolved into two or more peaks of radioactivity. Medullary localized antigen banded in a region of the gradient rich in lysosomal enzymes and was considered to be present in vesicles. Antigen was not found in a region of the gradient rich in mitochondria. Antigen from lymphoid follicles of nodes or from spleen white pulp banded at high density values and was considered to be present as an antigen–antibody complex, possibly associated with membrane.

Equilibrium density centrifugation in Urografin gradients provides a means of separating and examining the properties of antigen in lymphoid tissues.

INTRODUCTION

When antigen labelled with radioactive iodine was injected into the hind footpads of rats, the draining lymph nodes (popliteal and aortic nodes) trapped some antigen, the proportion retained varying according to the chemical and physical properties of the antigen (Ada, Nossal and Pye, 1964a). If those nodes containing labelled antigen were pooled and fractionated in a standard sucrose medium, at all time intervals except shortly after injection, up to 70 per cent of the radioactivity was recovered in a 'large granule' (LG) fraction, which sedimented between 10,000 and 200,000 *g* minutes. In preliminary experiments this fraction was subjected to equilibrium centrifugation in gradients of sucrose but no separation of the various components in the fraction was achieved (Ada and Williams, 1966). Autoradiography of sections of lymph node containing labelled antigen indicated that the antigen was in macrophages present in the medulla of the node and also in primary lymphoid follicles which occur in the node cortex (Nossal and Ada, 1963). High resolution autoradiography subsequently showed that antigen in medullary macrophages was present in the cell cytoplasm in vacuoles

often associated with lysosome-like particles; by contrast antigen in the lymphoid follicle in the cortex appeared to be associated with the surface membranes of reticulum cells and rarely to be inside such cells (Mitchell and Abbot, 1965).

This paper reports experiments in which the LG fractions from antigen-containing lymphoid tissues have been further fractionated by equilibrium centrifugation in gradients of Urografin. One or two peaks of radioactivity (antigen) were obtained in such gradients and evidence is presented which indicates that one peak of radioactivity represented antigen derived from medullary macrophages and that the other peak represented antigen derived from follicles.

MATERIALS AND METHODS

The following procedures have been described in detail in the preceding papers (Ada and Williams, 1966; Williams and Ada, 1967): preparation of tissue homogenates and isolation of LG fraction; conditions of equilibrium centrifugation in gradients of sucrose, dextran-40 and Urografin; removal of fractions and estimation of enzymes.

In brief, tissues were homogenized in buffered sucrose (0·29 M) and the fraction sedimenting between 10,000 and 200,000 *g* minutes (the LG fraction) isolated. After resuspension, this fraction was layered on top of the sucrose gradient (density range 1·05–1·20), dextran-40 gradient (density range 1·05–1·18) or on top of, or underneath, the Urografin gradient (density range 1·05–1·25). Gradients were centrifuged for 16 hours at 37,020 rev/min in the SW-39 rotor of the Beckman Model E ultracentrifuge.

Antigens

Particulate flagella, and soluble and polymerized flagellin from *S. adelaide* were prepared as described previously (Ada, Nossal, Pye and Abbot, 1964b). Human serum albumin (HSA) was a preparation from Behringwerke. These substances were labelled with carrier-free preparations of ^{125}I (Ada *et al.*, 1964a). In some experiments radioiodinated HSA solutions at a concentration of 0·01 per cent (w/v) in phosphate buffered saline, pH 7·3, were heated at 90° for 15 minutes prior to injection.

Immediately after labelling with isotope, antigens were injected either intravenously or into the hind footpads of young adult Wistar rats. Doses were 1–2 μg of bacterial antigen, 10 μg HSA and 50 μg rat IgG.

For the purpose of simplicity, the presence of radioactivity in a fraction is taken to indicate the presence of antigen, except where otherwise mentioned.

RESULTS

Interpretation of the results of equilibrium centrifugation experiments in density gradients has been facilitated by the use of LG preparations from rat lymph nodes or spleen, in which it was previously shown by autoradiographic examination that the antigen was preferentially or exclusively localized in the medulla or in the lymphoid follicles of the lymph node or in the red or white pulp of the spleen.

Experimental situations were investigated in which the following were used as antigen:

(1) *A solution of HSA denatured by heating*: After injection into the hind footpad the protein was almost entirely localized in medullary macrophages in the popliteal and aortic

lymph nodes. Trapping of antigen in these nodes reached a maximum value at about 24 hours after injection (Lang and Ada, 1967).

(2) *HSA and rat anti-HSA serum mixtures*: After injection of such a complex into the hind footpad up to thirty-five times more antigen was trapped and retained in the lymph nodes, compared with retention of HSA injected alone. Twenty-four hours after injection autoradiographic examination showed that antigen was present in large amounts in lymphoid follicles but was also present in the medullary macrophages. By 3 days after injection the lymphoid follicles of the node still contained large quantities of antigen but there was very little present in the medulla (Ada and Lang, 1966).

(3) *Polymerized flagellin*: After intravenous injection of polymerized flagellin there was early appearance of antigen in the red pulp and in the marginal zone between the red and white pulp of the spleen. The red pulp cleared progressively and by 8 hours was virtually unlabelled. During this time label appeared in the white pulp and progressively disappeared from the marginal zone until the germinal centre was heavily labelled (24 hours after injection). Thereafter germinal centre cap labelling persisted but marginal zone labelling disappeared (Nossal, Austin, Pye and Mitchell, 1966). Following this investigation the technique was applied to several other systems.

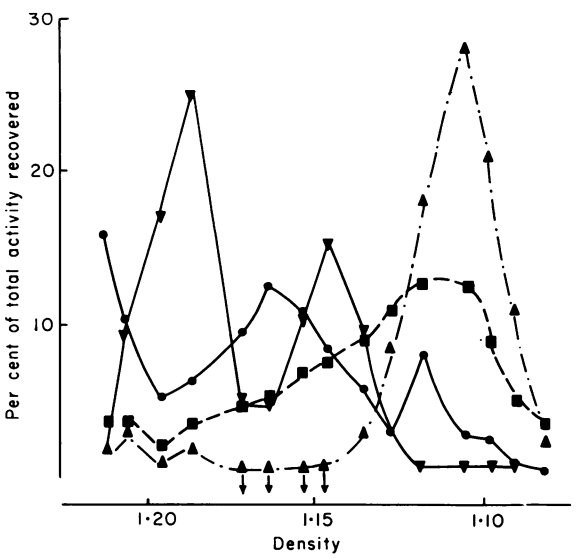

FIG. 1. Distribution of enzyme activities after centrifugation of a lymph node LG preparation in a Urografin gradient. ▲, ATPase; ▼, succinate dehydrogenase; ■, acid phosphatase; ●, β-glucuronidase.

CENTRIFUGATION OF LG FRACTIONS IN GRADIENTS OF SUCROSE OR DEXTRAN

It was previously shown that when nodes of rats injected 1 or 7 days previously with labelled flagella were fractionated, and the crude extract obtained submitted to equilibrium centrifugation in a sucrose gradient, only one band of opacity coincident with one peak of radioactivity, density $1{\cdot}142 \pm 0{\cdot}017$, was found (Ada and Williams, 1966). Similar results were obtained when HSA was used as antigen, irrespective of whether the antigen was localized apparently exclusively in the medulla (heated HSA injected)

or preferentially in the lymphoid follicles (HSA+antibody injected). This occurred whether a crude extract or the isolated LG fraction of the node was centrifuged. That is, despite the indication from autoradiography that antigen was associated with two different types of cellular particles, there was only one peak of radioactivity and of opacity. In a medium such as sucrose to which isolated vesicles are permeable, vesicles and membranes might be expected to equilibrate at the same density. It was considered that if a high molecular weight solute were to be used to form the gradient, vesicles should be impermeable to this solute and would equilibrate at a lower density than would isolated membrane.

These experiments were, therefore, repeated using gradients of dextran instead of sucrose. However, whether flagella, heated HSA or HSA+antibody preparation had been injected into the rats, centrifugation of the LG fraction from rat lymph nodes resulted in

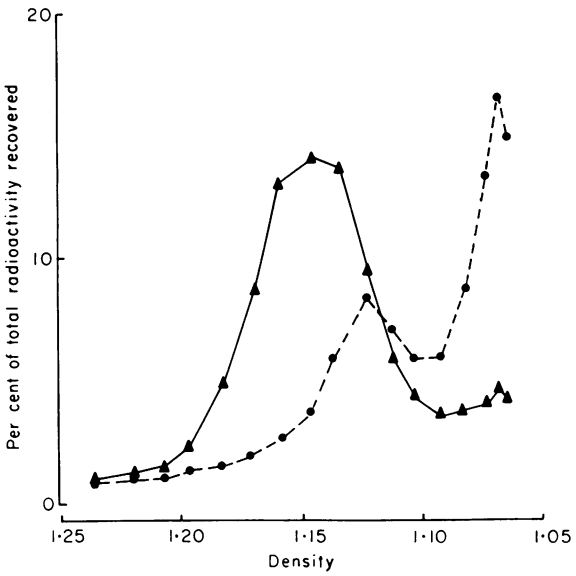

FIG. 2. Distribution of radioactivity after centrifugation in Urografin gradients of LG preparations from rats injected 24 hours previously with denatured HSA. ▲, LG preparation; ●, LG preparation frozen and thawed before centrifugation.

only one peak of radioactivity. This band was found at a density $1{\cdot}108 \pm 0{\cdot}007$ in gradients of dextran which varied between 1·04–1·12 and 1·04–1·18. The density at which the radioactivity equilibrated had been decreased as expected, but there was still only a single peak of radioactivity.

From these results it appeared possible that in both these media complexing between particles was occurring. Some separation of particulate components in the LG fractions of tissues had been achieved by centrifugation in gradients of Urografin (Williams and Ada, 1967). The main part of this paper is concerned with the analysis of antigen distribution after equilibrium centrifugation of the LG fractions from antigen-containing lymph nodes and spleen in Urografin gradients. The distribution of enzyme activities in rat node LG preparations after centrifugation is shown in Fig. 1. A similar distribution was found in the spleen LG fraction (Williams and Ada, 1967).

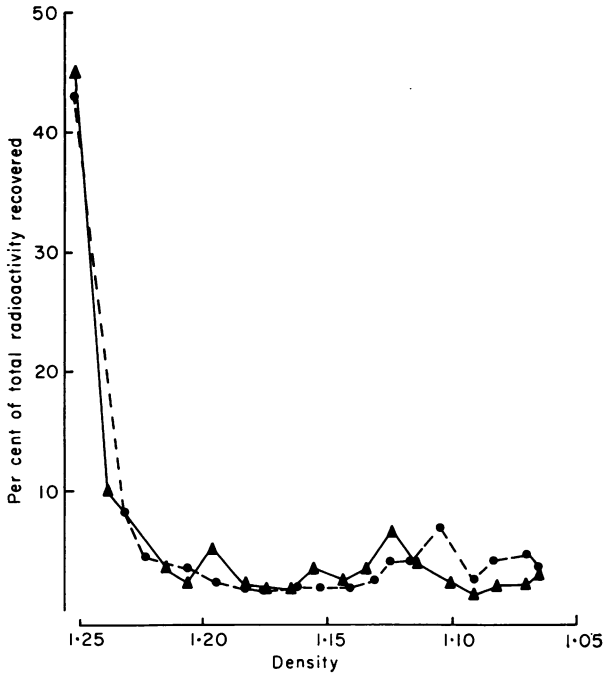

FIG. 3. Distribution of radioactivity after centrifugation in Urografin gradients of LG preparations from rats injected 24 hours previously with HSA–antibody complex. ▲, LG preparation; ●, LG preparation frozen and thawed before centrifugation.

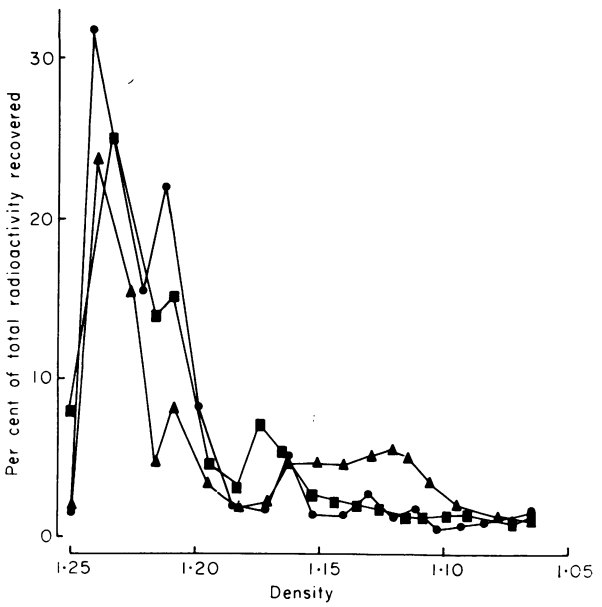

FIG. 4. Distribution of radioactivity after centrifugation in Urografin gradients of LG preparations from spleens of rats injected with polymerized flagellin. ▲, 2 hours after injection; ■, 24 hours after injection; ●, 48 hours after injection.

CENTRIFUGATION OF LG FRACTIONS IN GRADIENTS OF UROGRAFIN

After the injection of denatured HSA

If an LG fraction from the nodes of rats which had been injected with heated HSA 24 hours earlier was placed at the top of a Urografin gradient and centrifuged to equilibrium, the distribution of antigen throughout the gradient was as shown by the solid line in Fig. 2. If the LG fraction was placed underneath the gradient and then centrifuged to equilibrium the distribution of antigen was similar except that the peak of radioactivity was sometimes at a higher density level (up to 0·015 unit displacement), and a small amount (<5 per cent) of presumably unbound antigen which formerly remained at the top of the gradient was now at the point of insertion, i.e. underneath the gradient. If the LG fraction was frozen and thawed, placed on the top of the gradient and then centrifuged to equilibrium, the distribution of antigen was as represented by the dotted line in Fig. 2. Two changes occurred: (1) a large proportion (~40 per cent) of the radioactivity was now non-sedimentable, remaining above a density of 1·10; and (2) most of the radioactivity in the density range 1·10–1·18 now banded more sharply at the value 1·125. A similar pattern of antigen distribution was found at various times between 4 and 48 hours after injection of denatured HSA into rats.

After the injection of HSA–antibody complex

If an LG fraction from the nodes of rats which had been injected with HSA+antibody 24 hours earlier was placed at the top of the Urografin gradient and centrifuged to equilibrium, the distribution of antigen throughout the gradient was as represented by the solid line in Fig. 3. About 65 per cent of the radioactivity sedimented to density value greater than 1·20 density. There was a slight but reproducible peak at density 1·195 (this was only obtained when a sample was introduced at the top). The remaining radioactivity was distributed over the density region 1·10–1·18 with a shallow peak at about 1·13. A similar distribution was found if the sample was introduced underneath the gradient but with a slightly higher proportion of radioactivity recovered at a density >1·20. If the LG fraction was frozen and thawed, placed on top of the gradient and centrifuged to equilibrium, the distribution of radioactivity obtained is shown by the broken line in Fig. 3. Four points can be noted: (1) the proportion of radioactivity sedimenting to a density greater than 1·20 was unchanged, (2) the small peak of radioactivity at 1·19 had disappeared, (3) the radioactivity in the density range of 1·10–1·18 now banded at a density of 1·120 (previously 1·130), and (4) the proportion of radioactivity remaining at a density <1·10 was about 5 per cent higher than previously. A similar distribution of antigen was seen at time points between 1 and 5 days after injection of HSA+antibody into rats.

After the injection of polymerized flagellin

In these experiments rats were injected intravenously with 10-μg amounts of labelled polymerized flagellin, the rats killed 2, 24 and 48 hours later, the spleens removed, and samples taken for autoradiography and fractionation. The LG fractions were placed on top of the Urografin gradient, centrifuged as before and the fractions collected. Fig. 4 shows the pattern of radioactivity distribution at these time points. In each case there were two peaks in the density region 1·18–1·25 (in separate experiments this differentiation

was not found in fractions from lymph nodes containing polymerized flagellin). In spleen the pattern obtained in 2 hours also showed a broad peak of radioactivity in the 1·10–1·18 density region. At later time points this broad band was less evident, but there was a progressive appearance of a peak of radioactivity at a density of 1·16–1·17. If the fractions were frozen and thawed before centrifugation: (1) there was no nett loss of radioactivity from the density region of 1·18–1·25 but only one peak in the region was observed; and (2) there was a small nett loss of radioactivity from the density region 1·10 to 1·18, and the peak of radioactivity in the density region 1·16–1·17 was shifted to a slightly lower density (cf. the behaviour of fractions from nodes containing HSA+antibody).

INTERPRETATION OF RESULTS

Three considerations led us to believe that in these three systems the radioactivity found in a density range 1·10–1·18 was associated with subcellular particles which came from medullary macrophages.

(1) Autoradiography of nodes from rats injected with heated HSA showed almost complete localization of antigen in these cells. In the Urografin gradient at least 80 per cent of the radioactivity was in this density range.

(2) Two lysosomal enzymes were present in largest amounts in this density range. This was consistent with the autoradiographic evidence that antigen in these cells was in vacuoles associated with lysosome-like particles.

(3) Submission of the LG fraction to cycles of freezing and thawing liberated a large proportion of the radioactivity as non-sedimentable material. This would be expected if the vesicles contained antigen in an unbound form (Ada and Williams, 1966). Similarly, the radioactivity which was present at density values greater than 1·18 was considered to come from the follicular area of the node for the following reasons.

(1) Negligible proportions were seen in those cases where to judge from autoradiography there was no, or minimal, follicular localization.

(2) Sedimentation of the material was not affected by freezing and thawing, and this behaviour would be expected of membrane-bound material or antigen–antibody complexes.

INVESTIGATION OF ADDITIONAL SYSTEMS

This technique was then applied to several additional systems. Rats were injected in the hind footpads with: (1) labelled flagella, (2) labelled flagellin, and (3) labelled homologous IgG and the density distribution of antigen recovered in the LG fraction of the lymph nodes determined.

CENTRIFUGATION OF LG FRACTIONS IN GRADIENTS OF UROGRAFIN

After injection of flagella

The entry of labelled flagella into the popliteal and aortic lymph nodes after footpad injection of rats has been previously examined by standard autoradiographic procedures (Nossal, Ada and Austin, 1964). After appearing initially in the subcapsular sinus of the node the antigen was found shortly afterwards in the medullary macrophages, but was not detected in the lymphoid follicles until about 2 hours after injection. Antigen was not

present at any time in appreciable amounts in other areas of the node. Thereafter antigen was always present in both lymphoid follicles and medullary macrophages but as judged by the visual appearance of autoradiographs, the distribution of antigen between these areas was variable. In the present experiments autoradiography was carried out on sections of some nodes to verify that antigen was present in follicles and medulla.

Table 1 shows the percentage of injected radioactivity present in the lymph nodes and the amount of radioactivity which was recovered in the LG fraction. The proportion of radioactivity in the 1·18–1·25 and in the 1·10–1·18 density regions of the gradient after centrifugation was estimated and this enabled the relative amounts of membrane-bound antigen and antigen in phagocytic vacuoles to be calculated. At all times, more radioactivity was present in the lower than in the higher density region and this ratio remained remarkably constant (Table 1, last line).

TABLE 1

DISTRIBUTION OF RADIOACTIVITY IN LYMPH NODE FRACTIONS FROM RATS PREVIOUSLY INJECTED WITH LABELLED FLAGELLA

Per cent radioactivity injected	Time after injections (hours)						
	1	2	4	16	24	72	168
In popliteal and aortic nodes	1·00	0·38	0·96	0·62	0·57	0·27	0·14
In LG fraction	0·32	0·12	0·28	0·27	0·26	0·14	0·07
After centrifugation							
At density >1·20	0·032	0·016	0·050	0·027	0·026	0·013	0·007
Density region 1·10–1·20	0·195	0·080	0·174	0·197	0·185	0·106	0·050
Radioactivity at density 1·10–1·20 : radioactivity at density >1·20	6·1	5	3·5	7·3	7·1	8·1	7·1

As was observed previously in the case of denatured HSA in lymph nodes, freezing and thawing of the LG fraction from nodes containing flagella: (1) released a significant proportion of radioactivity in a non-sedimentable form; and (2) the residual radioactivity in the density region 1·10–1·18 now peaked at a density of 1·12 in contrast to the main peak density of 1·141 found before freezing and thawing.

When the LG fraction was centrifuged after insertion of the sample underneath the gradient 10–20 per cent of the radioactivity was recovered in the density region <1·10. Figures for radioactivity in this region after centrifugation of node extracts containing heated HSA, HSA+antibody or spleen extracts containing polymerized flagellin were much lower (2–10 per cent). At present no explanation can be offered for the high figures in the flagella experiments.

After injection of flagellin

Autoradiography of rat nodes removed 24 hours after footpad injection of labelled flagellin showed that as was the case with flagella, there was a variable distribution of label between the medulla and lymphoid follicles. Equilibrium density centrifugation of the LG fraction from similar nodes showed that 9 per cent of the radioactivity was in the density region of 1·18–1·25 and 75 per cent in the region 1·10–1·18.

After the injection of rat IgG

Homologous labelled rat IgG localized in the medulla and in the lymph follicles of the draining lymph node after footpad injection (Ada, Nossal and Austin, 1964; Lang and Ada, 1967). Such nodes removed from rats 1–3 days after injection of the globulin were fractionated as usual and the LG fraction centrifuged. Seventy-five per cent of the radioactivity was recovered in the density region 1·10–1·18 and half of this was released in a non-sedimentable form after freezing and thawing. Less than 5 per cent of the radioactivity was recovered in the density region 1·18–1·25.

CENTRIFUGATION OF ANTIGEN ALONE OR OF ANTIGEN MIXED *in vitro* WITH AN LG FRACTION FROM SPLEEN OF UNINJECTED RATS

Soluble antigen preparations, such as flagellin, γ-globulin or denatured HSA, did not sediment appreciably when centrifuged under these conditions. Particulate antigen preparations such as flagella or polymerized flagellin did sediment but the pattern of sedimentation was different from that observed with *in vivo* preparations of antigen in lymph node fractions. This difference was demonstrated for example when preparations of polymerized flagellin were mixed with either an LG preparation from rat spleen or with normal rat serum and the rate of sedimentation compared with that of antigen bound to the LG fraction of the spleen after intravenous injection of the polymerized flagellin (Table 2).

TABLE 2

RATE OF SEDIMENTATION OF POLYMERIZED FLAGELLIN IN GRADIENTS OF UROGRAFIN

Time of centrifugation (hours)	Form of antigen	Percentage of radioactivity in the density region		
		1·18–1·25	1·10–1·18	1·05–1·10
1½	In LG fraction *in vivo**	10·5	74·7	14·9
	With LG fraction *in vitro*†	2·1	11·5	86·5
	With 10 per cent (v/v) normal rat serum	2·1	4·1	93·5
3	In LG fraction *in vivo*	48·8	40·4	11·1
	With LG fraction *in vitro*	6·6	10·8	82·7
	With 10 per cent (v/v) normal rat serum	4·3	1·3	94·1

* Rats were killed 18 hours after the i.v. injection of labelled polymerized flagellin and the LG fraction prepared from a spleen extract.

† Labelled polymerized flagellin (100 ng) was mixed with a spleen LG fraction and centrifuged.

Although polymerized flagellin when mixed *in vitro* with a spleen LG fraction did sediment in this density gradient, sedimentation of radioactivity in the *in vivo* preparation occurred more rapidly and more completely as though complex formation had occurred. Some binding did occur *in vitro* between antigen and some components of the LG fraction which banded in the density region 1·10–1·18. Pre-incubation of the LG fraction for 1 hour, at 37°, prior to centrifugation slightly increased the extent of this binding. No binding of antigen occurred in particles which banded at a density of 1·18 (mitochondria).

DISCUSSION

Attempts to separate antigen-containing subcellular components have been carried out for a number of reasons.

(1) To confirm the results of high resolution autoradiography showing the type of particles to which the antigen was bound.

(2) To allow the state of antigen associated with each component to be studied.

(3) To isolate the particular subcellular fractions concerned so that reactions between them and antigen *in vitro* might be studied.

These aims have been partly achieved. The work reported in this paper continues from the earlier observation that antigen trapped in a draining lymph node after footpad injection into rats was largely recovered in the LG fraction of tissue homogenates. Antigen not recovered in this fraction was considered to be in a free or degraded state in the cell cytoplasm, in cell nuclei or in unbroken cells (Ada and Williams, 1966). The basic finding of the present work was the demonstration that antigen present in the LG fraction could be further separated into two broad categories, and this separation paralleled the type of localization in the lymphoid organ as demonstrated by radioautography. The fractionation was achieved by means of equilibrium centrifugation in gradients of Urografin, density range 1·05–1·25. Conditions were chosen which allowed true equilibrium to be obtained (Williams and Ada, 1967).

Antigen present in the LG fraction was distributed in three density regions, 1·18–1·25, 1·10–1·18 and 1·05–1·10. Antigen in the density region 1·05–1·10 represented free or 'released' material and, except for experiments with flagella or after intentional disruption of particles prior to centrifugation, the proportion of antigen in this region was negligible. The cellular derivation of antigen present in the other density regions was investigated by correlating their position in the gradient with results of autoradiography carried out on tissue sections. Three model systems, in which antigen was known to be almost exclusively present in the medulla or in lymphoid follicles, were investigated. Recovery of antigen in the density region 1.10–1·18 correlated with the presence of antigen in the medulla of lymph nodes, whereas the finding of antigen in the density region 1·18–1·25 correlated with the presence of antigen in the follicles of nodes or white pulp of spleen. When three other systems in which autoradiography showed that antigen was distributed between the node medulla and follicles were investigated, there was again a separation of antigen into different density regions, the percentage in the high density region varying from 20 per cent (flagella as antigen) to <5 per cent (γ-globulin as antigen).

Several results suggested that antigen recovered in the density region 1·10–1·18 was derived from particles which corresponded to the antigen-containing vacuoles seen in electron-micrographs of medullary macrophages. Two lysosomal enzymes banded in this density region. The association of antigen with such granules in the gradient was specific, as in no case was antigen found in the density region (1·18) where mitochondria were most abundant. In every case, antigen present in the 1·10–1·18 density region was in particles which could be disrupted, liberating some radioactivity in a non-sedimentable and, in those cases investigated (Ada and Williams, 1966), low molecular weight form. The antigen-containing structure left after the disruptive process equilibrated at a density close to that of ATPase, the marker used for membrane. Prior to disruption, the radioactivity was spread fairly uniformly over this density range, suggesting a heterogeneity of the antigen-containing particles. Some of these may have been phagolysosomes (Straus, 1963)

but others may have acted as 'storage granules' for antigen. With flagella, it was shown that up to 60 per cent of the radioactivity in the LG fraction was associated with specific antigen (Ada and Williams, 1966). In the present experiments centrifugation in Urografin of a similar LG fraction prepared from rat lymph nodes removed 7 days after injection of labelled flagella gave a distribution of radioactivity which suggested that about 80 per cent of the radioactivity had been present in medullary macrophages. These two results when considered together suggested that some radioactivity present in the medulla was still associated with specific antigen—i.e. that while portions of the antigen might be broken down in medullary macrophages some may be sequestered there. By contrast, denatured HSA or the HSA–antibody complex was not retained for long periods in the medulla.

Antigen recovered in the highest density region sedimented there because of the formation of a large complex, probably with antibody. It is possible that when soluble preparations (flagellin, IgG) were used as antigen, some membrane-associated antigen may not have reached equilibrium and would have been recovered in a lower density region. ATPase activity was not detected in the high-density complex but this may have been due to the small amount of membrane present or to the lack of ATPase in the membrane of the reticular cell. The complex was not affected by freezing and thawing; this would be expected whether or not membrane formed part of the complex. The results which most suggested that membrane might form part of this complex was the finding of two peaks of radioactivity in the high density region after centrifugation of spleen LG preparations from rats injected with polymerized flagellin. This separation into two peaks was most prominent at a time when autoradiographic examination showed two clearly separate zones of antigen localization in the white pulp area of the spleen, as though different cells were involved. However, direct biochemical proof of complexing of antigen with the reticular cell membrane was not obtained.

In essence the centrifugation procedure separates antigen present inside vesicles from antigen present as an antigen–antibody complex on cell membranes. Provided further high-resolution autoradiographic studies continue to show that these types of binding characterize antigen localized in medulla and follicles, then the centrifugation procedure supplements and is more quantitative than autoradiographic examination of antigen distribution in lymphoid tissues.

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