

The Uptake and Localization of Proteins, Evans Blue and Carbon Black in the Normal and Pathological Thymus of the Guinea-Pig

J. N. BLAU AND N. VEALL

Departments of Pathology and Medicine, Guy's Hospital Medical School, London, S.E.1

(Received 25th March 1966)

Summary. Radioiodine-labelled homologous serum albumin and heterologous γ -globulin reached the extravascular space of the guinea-pig thymus in concentrations comparable with those in the spleen and in lymph-nodes. The uptake of proteins in a locally X-irradiated involuting thymus gland was approximately doubled at 6, 12, 24 and 48 hours compared with non-irradiated controls. At 7 days the concentration was greater than in the other lymphoid tissues examined, namely spleen, cervical and mesenteric lymph-nodes, and was greater than in control glands by a factor of two to four.

Localization of foreign material was studied with Evans Blue and carbon black which were found in macrophages of the capsule, cortex and medulla, and in Hassall's corpuscles. The foreign material showed a marked increase in the parenchyma of glands during involution whether produced by X-irradiation, cortisone or corticotrophin.

The significance of these findings in relation to the formation of germinal centres in the thymus in pathological conditions is discussed.

INTRODUCTION

The questions posed in this investigation were: 'Why does the normal thymus fail to form germinal centres like the spleen and lymph-nodes? Is it because antigens do not reach the gland in adequate concentration, i.e. a blood-thymus barrier as proposed by Marshall and White (1961), or do antigens arrive in the thymus to find the wrong milieu there?'

In an attempt to answer these questions, quantitative comparisons were required using different sized molecules. Up to the present quantitative studies have been limited: the entry of ^{35}S -labelled bovine serum albumin (BSA) into the thymus of newborn and immunologically tolerant rabbits was studied by Garvey, Eitzman and Smith (1960). Clark (1964), working with mice, measured the radioactivity of ^{125}I -labelled human serum albumin (HSA) in whole lymphoid organs, including the thymus. He found that they were all equally radioactive. Qualitative studies have been more extensive and recent reports include those of Kaplan, Coons and Deane (1950), Green and Bloch (1963), Kostowiecki (1963) and Clark (1964). A variety of antigens were injected subcutaneously or into the circulation.

Most investigators have studied the normal thymus. In the present study a comparison of the behaviour of normal and acutely involuting glands has been made. This may be

relevant to the study of human disease, since the thymus in man begins to involute at puberty and diseases in which the thymus is implicated most commonly occur in adult life. Of particular interest is the thymus in myasthenia gravis where the gland frequently contains germinal centres (Castleman, 1960). In this investigation tissue uptake of two types of protein, labelled with ^{125}I , has been measured: homologous serum proteins (HSP) of the guinea-pig, to give a measure principally of albumin, and a foreign protein, human γ -globulin (HGG). A second tracer (^{131}I -labelled HSA or polyvinylpyrrolidone (PVP)) was used to determine the plasma volume of the tissue so that the extravascular labelled protein uptake per gram of tissue could be determined by difference. Evans Blue and carbon black were used as models of soluble and particulate antigens and the localization of these substances was studied in other groups of animals under normal and pathological conditions.

MATERIALS AND METHODS

Animals

Albino guinea-pigs (Hartley strain) of both sexes weighing between 230 and 630 g were grouped into animals of comparable weight and similar sex. Most of the animals weighed between 250 and 350 g. Each experimental animal had its own control. The guinea-pigs were fed on Diet S.G.1 pellets (Ranks) (supplied by J. Ranks Ltd, Deptford Bridge Mills, London, S.E.8.), fresh cabbage leaves and water *ad libitum*.

Involution of thymus glands

Involution of the thymus was effected by two methods in different groups of animals:

(a) *Local X-irradiation*. Animals were anaesthetized with intraperitoneal Standard Veterinary Pentobarbitone Sodium B.P. (Abbotts). The animals that were to be irradiated were lain on their backs in a Perspex box and their limbs secured with sellotape. A lead sheet of 3 mm thickness covered the animals apart from a window, 4×5 cm through which X-rays were directed to give a tissue dose of 300 r to the thymus. The constants of irradiation were 40 r/minute, 220 kV and half value layer equivalent to 1.4 mmCu.

(b) *Cortisone or corticotrophin*. Groups of animals received subcutaneous injections of Cortisone Acetate B.P. (Organon) 2.5 and 5.0 mg twice daily for 5 and 7 days respectively. Control animals had the same number of injections of sterile saline in similar volumes, 0.1 and 0.2 ml. Other animals had Corticotrophin Gelatin Injection, B.P. (Crookes), 4 units twice daily for 4 days and others for 7 days. Control animals had injections of normal saline.

Radioactive proteins

The proteins were iodinated with ^{125}I by the method of McFarlane (1958) and were injected by the intracardiac route within 1 hour of irradiation. The quantity of homologous serum protein (HSP) injected into each animal was 0.1–1.0 mg labelled with 10–25 μC of radioactive iodine and with human γ -globulin (HGG), 1–7 mg of protein labelled with 20–25 μC was used.

The HGG was a highly purified preparation (EG 137) supplied by the Blood Products Laboratory of the Lister Institute, and on paper electrophoresis contained 3.9 per

cent γ_1 - and 96.1 per cent γ_2 -globulin and was free from albumin, α - and β -globulin. On centrifugation it appeared as pure 7S γ -globulin.

Groups of animals were allowed to survive for periods of 6, 12, 24 and 48 hours, and others for 7 days. There were at least four animals, two irradiated and two controls in each group, although in some groups up to eight animals were included. In order to permit an estimate of the plasma content of the tissue specimens to be made, 1–2 μC of [^{131}I]HSA (RCC Amersham, Code No. IB.17P) or polyvinylpyrrolidone (Code No. IB.33P) were administered by intracardiac injection to each animal at the end of the appropriate survival period. Ten minutes later a 1–2 ml sample of blood for the determination of plasma activity was obtained by cardiac puncture and the animal was then killed with ether anaesthesia. Immediately after death both lobes of the thymus, a deep cervical and a mesenteric lymph-node, the spleen and a portion of muscle from a hind limb were taken. Each tissue and an aliquot of plasma were put separately into weighed and stoppered glass containers which were then weighed again.

The radioactivity of the plasma was compared with that of each tissue and counted in a well-type scintillation counter using a pulse height analyser set at 0.36 MeV for γ -rays of ^{131}I . The counts were then repeated with the analyser set at 25–35 keV for γ - and X-rays of ^{125}I . Cross-channel interference between the two isotopes was corrected by counting the appropriate standards in each channel. The extravascular uptake of the labelled protein (HGG or HSP) was calculated as follows:

$$\text{Plasma volume/g of tissue (ml)} = \frac{{}^{131}\text{I activity/g of tissue}}{{}^{131}\text{I activity/ml of plasma}}$$

Intravascular labelled protein (counts/min/g of tissue)

$$= {}^{125}\text{I activity/ml of plasma} \times \text{plasma volume of tissue.}$$

Extravascular labelled protein (counts/min/g of tissue)

$$= {}^{125}\text{I activity/g of tissue} - \text{intravascular labelled protein.}$$

Some of the animals had iodide in their drinking water to block thyroid uptake. This did not appear to affect the results. The amount of free ^{125}I in serum was checked in three groups of animals, four in each group, on the 1st, 2nd and 7th day after injection of the labelled protein and ranged from 1.0 to 1.8 with a mean of 1.3 per cent.

The tissues were fixed in 10 per cent formol saline, processed and stained with haematoxylin and eosin. In a few animals salivary gland was mistaken for the thymus and these animals were excluded. The results deal with thirty-three satisfactorily injected and surviving animals that had homologous serum proteins and thirty-one that had heterologous γ -globulin.

Evans Blue

Evans Blue (British Drug Houses) as a 2 per cent solution in saline was injected into the heart and some animals had additional injections into the peritoneal cavity. Some groups of animals were allowed to survive for 2 days and others for 7 days. The total quantity of dye injected ranged from 0.5 to 2.5 ml.

The tissues were examined histologically after staining with neutral red which gave a good contrast with the blue dye. Sections stained with haematoxylin and eosin were also examined. There were thirty-six animals in this group.

Carbon particles

Each animal in this group had one intracardiac injection of 0.1–0.15 ml/100 g body weight of indian ink (Günther Wagner Pelikan Werke, Hanover, Batch C11/1431a) which consisted of 10 per cent carbon with an average particle diameter of 200–500 Å stabilized with 4.3 per cent fish glue; and contained 1 per cent phenol in water. After injection the animals turned a slaty grey colour and returned to normal in a matter of minutes.

Histological sections of thymus, spleen, cervical and mesenteric lymph-nodes were stained with methylene blue. This provided good contrast for the carbon particles. In addition haematoxylin and eosin sections were also examined. There were forty animals in this group.

RESULTS

INVOLUTION OF THYMUS AFTER X-IRRADIATION

Weight of thymus and plasma volume of thymus

The wet weight of the thymus decreased progressively from 24 hours after irradiation. The weight of each irradiated and control gland has been plotted in Fig. 1.

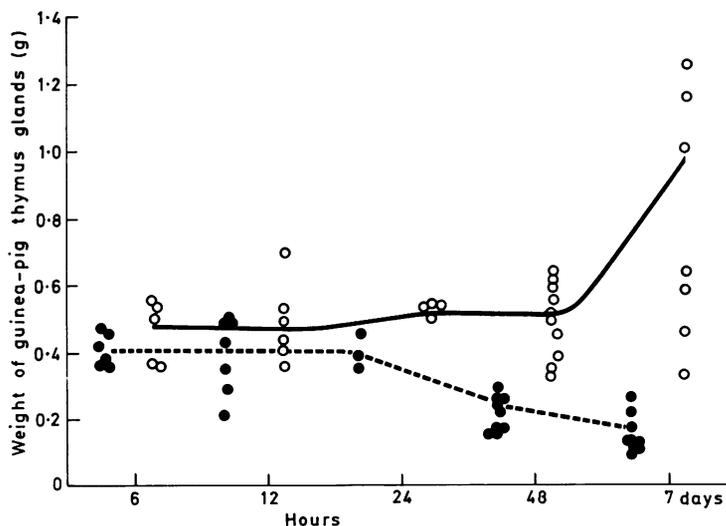


FIG. 1. Weight of guinea-pigs' thymus glands. ●, Irradiated; ○, unirradiated. The rapid loss in weight 2 and 7 days after irradiation is evident.

Six animals weighing 575–625 g had larger glands than the majority of the animals in the series and this accounts for the elevation of the weight curve of control glands at the 7-day interval. However, the weight of the glands of these large animals decreased markedly after irradiation and became equal to that of the irradiated glands of smaller animals.

The plasma volume of the irradiated thymus did not differ significantly from that of the control glands although at the 7-day interval there was a tendency for the values to be a little lower in the irradiated group. There was a marked variation in number and size of blood vessels to be seen in histological sections.

Concentration of extravascular homologous serum labelled proteins

Fig. 2 shows the uptake of iodinated serum proteins of the guinea-pig in the extravascular space of the spleen, cervical and mesenteric lymph-nodes, control and irradiated thymus glands and muscle at varying intervals after intravascular injection expressed in counts/min/g of tissue.

There was no significant difference between the uptake in cervical nodes which were in the same irradiation field as the thymus and the uptake in control animals. There was

TABLE I

MEAN VALUES OF EXTRAVASCULAR HOMOLOGOUS SERUM PROTEINS IN mg OF ALBUMIN/g OF TISSUE, UNCORRECTED FOR INCOMPLETE EQUILIBRATION

Time after injection	Thymus		Spleen	Cervical node		Mesenteric node	Muscle
	Irradiated	Unirradiated		Irradiated	Unirradiated		
6 hours	2.0(3)	1.9(3)	2.2(6)	4.5(3)	3.5(3)	4.1(6)	0.5(6)
12 hours	6.0(3)	1.9(3)	2.9(6)	7.8(3)	4.5(3)	6.1(6)	1.6(6)
24 hours	3.4(2)	2.5(2)	1.2(4)	4.9(2)	4.8(2)	4.6(4)	1.3(4)
48 hours	4.6(5)	2.0(5)	2.8(10)	5.7(5)	3.7(5)	4.0(10)	1.8(10)
7 days	7.8(4)	2.0(3)	3.0(7)	2.4(4)	3.5(3)	4.3(7)	2.3(7)

Figures in parentheses indicate number of animals examined.

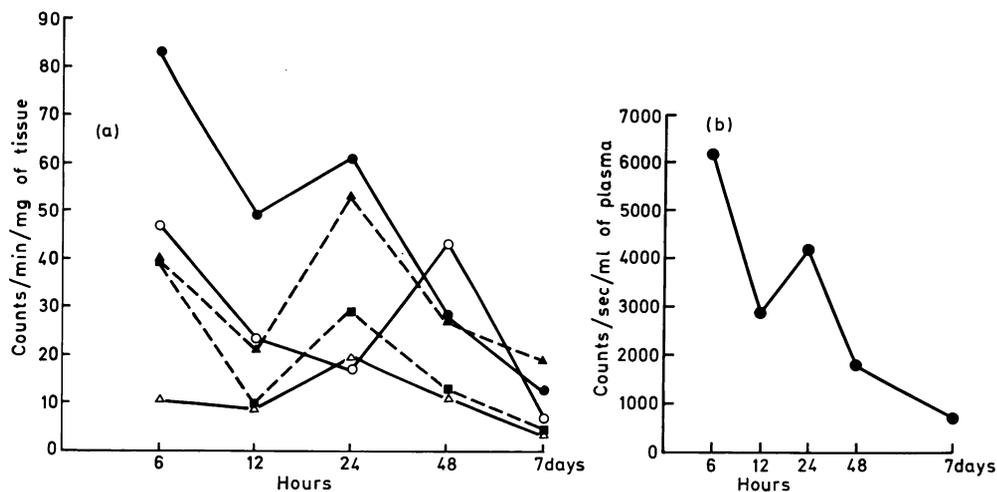


FIG. 2. Mean values of concentration of extravascular labelled homologous serum proteins in various lymphoid tissues and muscle at differing intervals after injection into the circulation. X-irradiation of the thymus was carried out less than 1 hour before injections. (b) is plotted from the mean values of the plasma radioactivity counts taken immediately before killing the animals. In (a): \blacktriangle , irradiated thymus; \bullet , cervical and mesenteric nodes; \circ , spleen; \blacksquare , unirradiated thymus; \triangle , muscle.

also no difference in uptake between cervical and mesenteric nodes. Consequently, the mean uptake values for the cervical, irradiated and control, and mesenteric nodes have been averaged and plotted together on the graph and are compared with the other lymphoid tissues in Fig. 2.

The extravascular concentration of labelled protein in the unirradiated thymus ran

closely parallel to that in the spleen and was less than that found in the lymph-nodes. The irradiated thymus had at all times a greater uptake of the labelled protein than the control glands and approximated to that found in lymph-nodes. The decrease in extravascular ^{125}I -labelled protein concentration in all tissues remained roughly proportional to the concentration in the plasma with the one exception of the irradiated thymus glands at the 7-day interval, when the labelled protein concentration was found to be about three times that of the control.

When the *extravascular content of radioactive albumin in the whole organ* was estimated, however, there was no difference between the control and the irradiated thymus glands.

Concentration of extravascular heterologous labelled protein

Fig. 3 shows the concentration of extravascular iodinated HGG in the spleen, cervical and mesenteric lymph-nodes, control and irradiated thymus glands and muscle at varying intervals after intravascular injection.

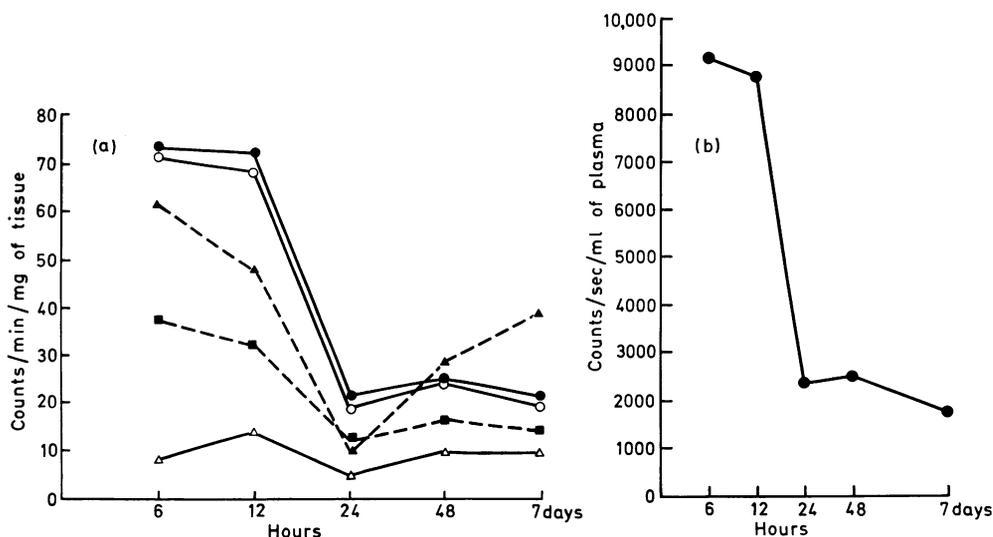


FIG. 3. Mean values of concentration of extravascular labelled human γ -globulin in various lymphoid tissues and muscle at differing intervals after injection into the circulation. X-irradiation of the thymus was carried out less than one hour before injections. (b) is plotted from the mean values of the plasma radioactivity counts taken immediately before killing the animals. In (a): ▲, irradiated thymus; ●, cervical and mesenteric nodes; ○, spleen; ■, unirradiated thymus; △, muscle.

As with homologous serum proteins there was no significant difference between the uptake in cervical nodes that had been exposed to X-rays and those not irradiated. There was also no difference in the uptake between the cervical and mesenteric nodes. The concentration of labelled protein in the lymph-nodes and spleen is seen to be similar throughout. The concentration in control thymus glands was lower than that in the other lymphoid tissues. By contrast the initial uptake in irradiated thymus glands was similar to that in the spleen and again there was a rise in concentration at the 7-day interval to nearly three times that of the unirradiated glands of control animals. This increase was comparable with that found using homologous serum proteins.

Again the total quantity of the extravascular labelled γ -globulin in the irradiated glands was no greater than that in the control glands, i.e. the difference was confined to the extravascular concentration.

TABLE 2
MEAN VALUES OF EXTRAVASCULAR HGG IN mg/g OF TISSUE, UNCORRECTED FOR INCOMPLETE EQUILIBRATION

Time after injection	Thymus		Spleen	Cervical node		Mesenteric node	Muscle
	Irradiated	Unirradiated		Irradiated	Unirradiated		
6 hours	1.4(3)	0.8(2)	1.6(5)	1.8(3)	1.1(2)	1.8(5)	0.2(5)
12 hours	1.2(3)	0.7(3)	1.6(6)	1.6(3)	1.9(3)	1.5(6)	0.3(6)
24 hours	1.8(1)	0.8(2)	1.8(3)	3.9(1)	1.1(2)	2.2(3)	0.5(3)
48 hours	2.4(4)	1.3(4)	2.0(8)	2.5(4)	1.9(4)	1.9(8)	0.8(8)
7 days	4.1(5)	1.5(4)	1.8(9)	2.7(5)	2.4(4)	1.9(9)	1.1(9)

Figures in parentheses indicate number of animals examined.

LOCALIZATION OF COLLOID AND PARTICULATE MATERIALS

In different groups of animals alternative methods of involution were used; irradiation, cortisone or corticotrophin. There was no difference in the overall picture and the results will be described together. More foreign material was seen inside the glands when longer time intervals were allowed after commencement of treatment.

Evans Blue

The thymus glands of treated animals were smaller and dark blue whereas control glands were pale blue. The cut surface of the lobes of the thymus were a uniform blue. In tissue sections, some capsular macrophages contained the dye whilst other proteins of the capsule were unstained. Adjacent to the stained areas of the cortex, a few cortical thymocytes were seen sometimes to contain dye in their cytoplasm. This applied to the thymuses of treated and control animals. However, in the involuting glands more dye was seen in the medulla of the gland and was localized in macrophages (Fig. 4) and in some Hassall's corpuscles (Figs. 5 and 6). The macrophages were situated perivascularly as well as away from blood vessels.

In control glands the distribution of the dye was similar but the intensity was less. The results with radioactive labelled albumin and Evans Blue differ: the lymphoid tissues showed similar concentration of protein, yet with the blue dye there was much greater staining in the spleen than in the thymus or lymph-nodes.

Carbon black

The black material had a patchy distribution in irradiated glands whilst in control thymus glands there appeared to be no staining at all apart from occasional streaks on the surface. The irradiated glands when cut showed discoloration distributed throughout the tissue although some areas were darker than others which now had a greyish colour contrasting with the pink of the untreated ones. In microscopic examination it appeared that some lobes had taken up more carbon than the adjacent ones. Carbon particles were seen in the connective tissue cells of the capsule and between lobules. Perivascular and medullary macrophages and Hassall's corpuscles contained variable quantities of carbon (Figs. 7 and 8).

The spleens of all the animals contained much more carbon than the thymus glands, even the irradiated ones. The amount of carbon in the thymus and lymph-nodes was comparable, even though some deep cervical nodes in the irradiated animals appeared darker macroscopically.

DISCUSSION

This investigation has demonstrated that molecules of varying sizes, including particulate matter, can reach the extravascular space of the thymus. Moreover, at 2-7 days the concentration of proteins with molecular weights of 60,000 and 150,000 whether homologous or heterologous, is similar to that found in the spleen. This is not surprising in view of the high concentration of γ -globulin found by Gitlin, Landing and Whipple (1953) and by White and Marshall (1962) in Hassall's corpuscles of infants, children, in normal adults and in those suffering from myasthenia gravis. In the latter investigation the globulin was shown to be predominantly of the 7S variety and was also to be seen in the germinal centres in the thymus of myasthenic patients. Similarly, for the smaller albumin molecule, Garvey *et al.* (1960) found that BSA injected subcutaneously had entered the gland and that the concentration in the thymus was one twenty-fifth to one and a half times that in the spleen, with a mean value of one-third. Saint-Marie (1963) was able to obtain immunofluorescent staining in the medulla and some areas of the cortex of the thymus of rats after injecting BSA into the mediastinum.

These experiments have also shown that as involution progresses and the gland shrinks, the protein is retained so that the concentration after 7 days exceeds that of the lymph-nodes and the spleen. The increase in albumin and globulin concentration in the irradiated thymus glands at 7 days as compared with the decrease in unirradiated thymus glands supports the view that there are no lymphatics inside the thymus. Clark (1963) in his electron microscopy studies was unable to find any evidence of lymphatic channels. Also Murray (1948) commented that unlike the irradiated spleen the cell debris which remained in the thymus following its irradiation was not immediately phagocytosed and clearance of necrotic material was delayed.

The entry of foreign substances is almost certainly by way of dilated blood vessels at the cortico-medullary junction and the entry of the carbon was visible by this route after X-irradiation and after systemic injection of cortisone (Blau, 1965).

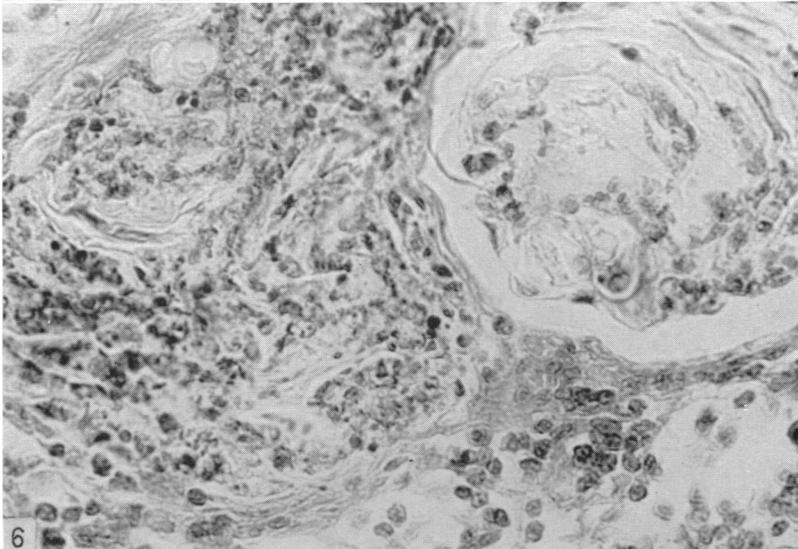
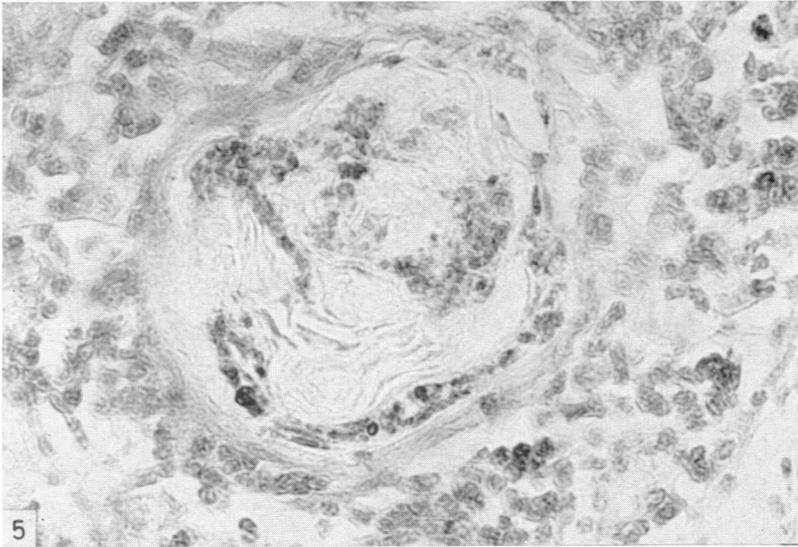
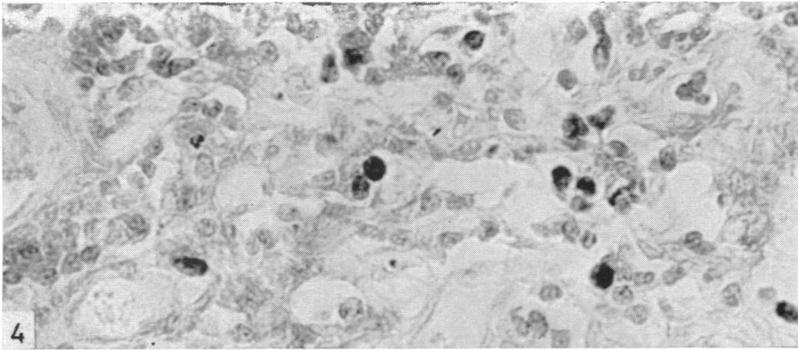
Perivascular collection of foreign material has been noted by a number of investigators (pneumococcal polysaccharide, Kaplan *et al.* (1950); heterologous serum albumin, Saint-Marie (1963)). However, Green and Bloch (1963) found a wide distribution of various particulate materials in many parenchymal macrophages: fifteen out of thirty-three adult mice showed staining with trypan blue and in newborn animals, fourteen out of thirty had carbon and seven out of thirty-one thorotrast in these cells. Ferritin was also demonstrated in macrophages deep in the parenchyma of the thymus of mice by

FIG. 4. Evans Blue is visible in macrophages in the medulla of the thymus. The macrophages are in the proximity of blood vessels. The thymus was irradiated and received a tissue dose of 300 r 7 days previously. 1.5 ml of 2 per cent Evans Blue injected intracardially within 1 hour of irradiation. Stained with neutral red. $\times 520$.

FIG. 5. Hassall's corpuscle from animal illustrated in Fig. 4 showing Evans Blue aggregated and taking up a laminar distribution at edge of corpuscle. $\times 520$.

FIG. 6. Compound Hassall's corpuscle from same animal illustrated in Figs. 4 and 5. One portion of the corpuscle is almost entirely free from the dye whilst the nucleated portion contains large amounts of the dye. $\times 520$.

Uptake and Localization of Proteins



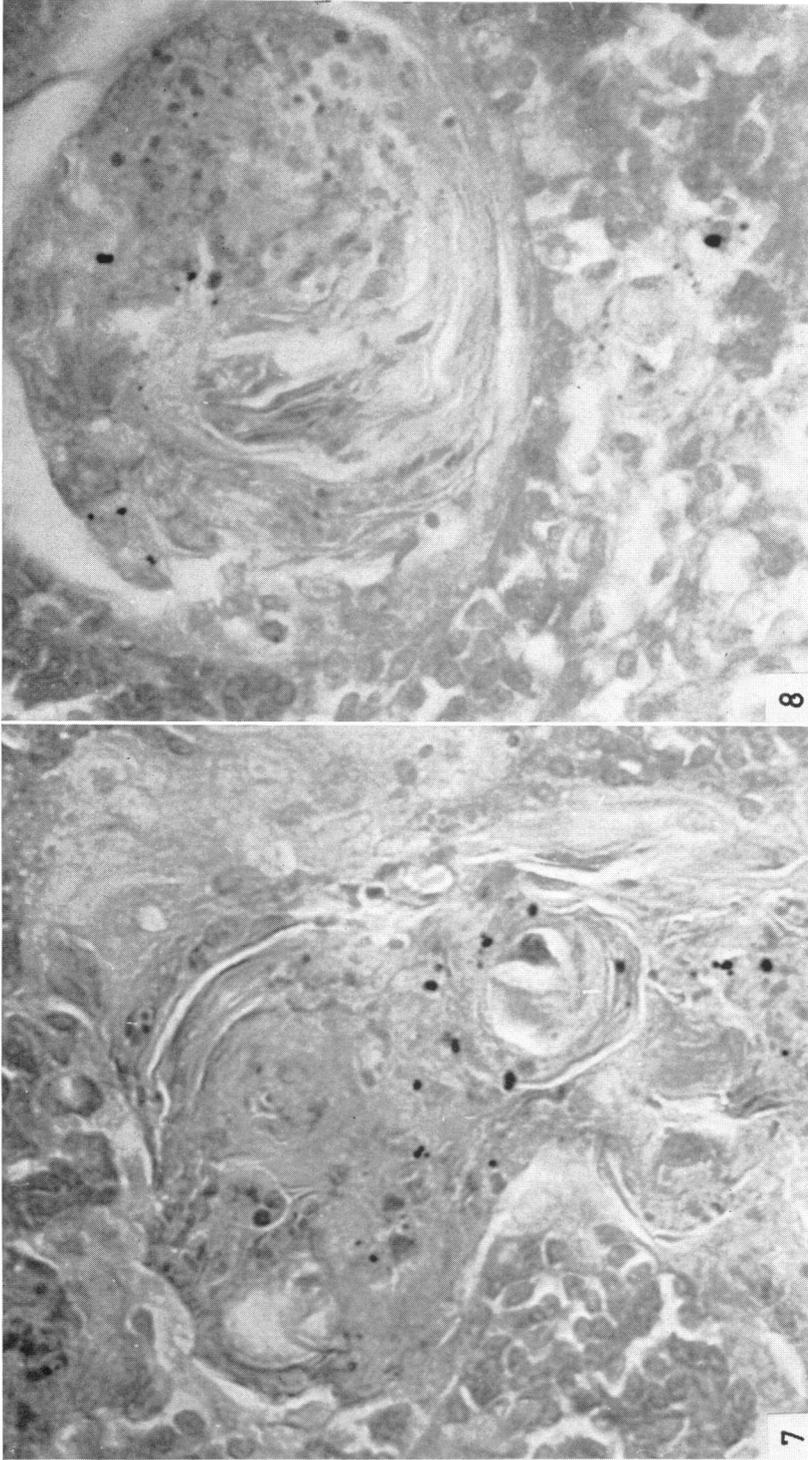


FIG. 7. Carbon black in Hassall's corpuscle. The particles are seen in the laminated as well as in the nucleated portion of the corpuscle. From an animal that had injections of 5 mg cortisone subcutaneously twice daily for 7 days and 0.3 ml indian ink injected into the heart immediately after the first dose of cortisone. Stained with methylene blue. $\times 740$.

FIG. 8. Carbon black in perivascular macrophage and in Hassall's corpuscle. The latter is predominantly nuclear. Same animal as Fig. 6. $\times 740$.

Clark (1964) with the electron microscope. All these findings confirm that larger molecules can, to some extent at least, penetrate into the parenchyma of the thymus.

Although the extravascular concentration of labelled albumin was of the same order in the thymus and spleen, when Evans Blue was used there appeared to be much more dye in the spleen than in the thymus as seen in the histological sections. Similarly, much less carbon was seen in the thymus than in the spleen. Even when the entry of carbon was enhanced after X-irradiation or cortisone, the concentration of the carbon was very small compared to the high density in the spleen. However, the concentrations of carbon in lymph-nodes and thymus seemed comparable.

The localization of these particles was seen to be in the capsule, the perivascular macrophages, in macrophages in the medulla and to a lesser extent in the cortex. The surprising finding was the aggregation of carbon in Hassall's corpuscles. This was also true for the Evans Blue and confirmed the observations of Kostowiecki (1963). This uptake was markedly increased in the involuting glands and is consistent with the results obtained with labelled proteins. Not all Hassall's corpuscles took up the carbon particles and in this respect some seemed to be 'active' and others 'quiescent'. In treated animals the Hassall's corpuscles were increased in size and number whilst in control animals far fewer corpuscles contained carbon and then in lesser quantities.

Since antigens, macrophages and lymphocytes are present together in the thymus, theoretically the conditions are right for the formation of germinal centres. Germinal centres can occur in those New Zealand Black (NZB) mice which spontaneously develop a haemolytic anaemia (Burnet and Holmes, 1962), in patients with myasthenia gravis (Castleman, 1960) and experimentally in guinea-pigs when the gland is injected directly with killed typhoid-paratyphoid bacilli (Marshall and White, 1961). The problem remains why germinal centres do not occur when the animal is immunized systemically. It could be that some antigenic molecules are too large to enter the thymus in sufficient concentration or that an additional factor or factors are required.

Our results suggest that involution of the gland causes an increase in concentration of the antigen by two means: increase in the permeability of the blood vessels and shrinking of the gland, so that the concentration of the antigens in the thymus reached similar levels to those in other lymphoid tissues. A further requirement for the production of germinal centres may be the presence of immunologically competent lymphocytes and in this respect thymocytes are not equivalent to circulating lymphocytes (Arnason, Janković and Waksman, 1962). On the other hand, cells from the circulation enter the thymus during the recovery phase after X-irradiation (Ford and Micklem, 1963). Perhaps the simultaneous presence of an adequate antigenic stimulus as well as that of immunologically potent lymphocytes are required for the formation of the germinal centres inside the thymus gland.

The questions raised at the beginning of this paper remain only partially answered. It is evident that antigens do reach the thymus in concentrations which in other lymphoid tissues result in antibody production and the formation of germinal centres. The fact that the thymus only rarely forms these follicles is possibly due to local conditions that prevail inside the thymus at that particular time. Since Evans Blue, carbon black and pneumococcal polysaccharide are to be found in Hassall's corpuscles, it does appear that antigens can localize in these bodies which are unique to the thymus. This could result in the antigens being rendered inaccessible to lymphocytes, so that the gland does not give rise to germinal centres and antibody formation.

It is perhaps significant that in myasthenia gravis where there is a very high incidence of germinal centres in the thymus, the disease is precipitated, or the established condition worsened, by a number of factors including pregnancy, lactation, severe physical or emotional stress. All these cause involution of the gland (Dougherty, 1952), probably mediated by an enhanced endogenous secretion of a wide variety of androgenic, oestrogenic and adrenocortical steroid hormones, the latter being the most potent.

ACKNOWLEDGMENTS

It is a pleasure to thank the Medical Research Council for a personal grant to one of us (J.N.B.), to Professor C. W. M. Adams, for hospitality in the Pathology Department of Guy's Hospital Medical School, to Professor C. B. Allsopp and Mr K. W. Twinn for X-irradiation, to Blood Products Laboratory of the Lister Institute for the purified human γ -globulin, to Mr Ronald Morgan for photography, to the Department of Medical Illustrations for the diagrams, and to Miss Lesley Kennedy for technical assistance.

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