# THE IMMUNOLOGICAL REACTIVITY OF THE THYMUS

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A NUMBER of previous studies (Fichtelius, 1957; Bjørneboe, Gormsen and Lundquist, 1947; Harris, Rhoads and Stokes, 1948; Maclean, Zak, Varco and Good, 1957; Askonas and White, 1956), indicate that the intact thymus gland shows very slight or no production of antibody in immunized animals and also that the gland does not undergo the histological changes observed in other lymphoid tissues as a result of the parenteral administration of antigens. In addition, Dixon, Weigle and Roberts (1957), observed a very slight transfer of secondary antibody response by thymic cells, and Thorbecke and Keuning (1953), reported a failure of antibody production in thymic explants from immunized animals and no histological changes in the intact thymus. The production of runt disease by thymic cells and the experiments of Stoner and Hale (1955) however, indicate that thymic cells possess full immunological competence.

This study was undertaken to clarify the reasons for these previous observations and more particularly, to relate them to the fact that the human thymus in myasthenia gravis may show changes suggesting an immune response.

## MATERIALS AND METHODS

*Animals*

Male and female guinea-pigs and rats were employed in these experiments, the former animal being particularly suitable owing to the completely separated bi-lobed structure and cervical situation of its thymus gland. Young animals of about 400 g. (guinea-pig) and 130 g. (rat) were used.

*Surgical procedures* were carried out under nembutal and ether anaesthesia in the guinea-pig and ether anaesthesia in the rat. Direct injection of antigens at multiple sites into the thymus was performed in the guinea-pig with a tuberculin syringe and fine needle on one lobe of the exposed gland, the other being used as a control. TAB vaccine (Wellcome) diluted to 200 million orgs. per ml., and aluminium phosphate precipitated diphtheria toxoid (PAPT), 100 Lf or 6 Lf per ml. were employed as antigens; 0·2 ml. of antigen being injected. Aluminium phosphate mineral carrier was estimated at 1·5 mg. or 70 μg. per injection. Two series of injections were given at a 2 or 3 week interval, the animals being killed at 5–9 days after the last injection. Local thymic injury in the guinea-pig was produced by a cautery incision across the lower pole of one lobe of the gland. The opposite lobe was used as a control in the subsequent vital staining.

*Vital staining* was carried out on guinea-pigs and rats, both on the intact animal and in animals with thymic injury. A dose of 2 ml. of 2 per cent trypan blue (Revector) by subcutaneous injection or Evans blue (Gurr) was employed in the rat, and 8 ml. 1·5 per cent trypan blue per 400 g. body weight in the guinea-pig; the animals being killed 3–6 days after injection. Some animals were given a second injection after an interval of 2–3 days. In animals with thymic injury the dye was injected 30 min. after its infliction.

*Localization of Injected Antigens and Antibody by the Fluorescent Antibody Technique : Polysaccharide and Protein Test Antigens*

The following materials were injected by the external jugular vein in guinea-pigs and by the femoral vein in the rat. In guinea-pigs in which cautery injury had been inflicted on one lobe of the thymus, injection was made 10 min. after injury, and was normally made on the side opposite to that of the injured thymus lobe.

Streptococcus pneumoniae *type III capsular polysaccharide.*—Rats of 140 g. were injected with 10 mg. in 0·5 ml. of physiological saline. Guinea-pigs of 400 g. were injected with 12 mg. in 1·0 ml. of saline. These animals were killed 2 or 4 days after injection.

*Diphtheria antitoxin.*—Pepsin refined globulin prepared in the horse, provided by Burroughs Wellcome Ltd., 3300–5000 i.u. per animal.

*Preparation of tissue sections*

*Localization of the capsular polysaccharide of* streptococcus pneumoniae *type III.*—Portions of thymus, lymph nodes, spleen, and liver were fixed for from 18 hr. at room temperature to 48 hr. at 5° in a 65 per cent alcohol-10 per cent formalin mixture. Tissues were passed directly into 70 per cent alcohol, dehydrated and embedded in wax. Sections (5 $\mu$) were floated out on warm 70 per cent alcohol and mounted avoiding all contact with water. After dewaxing in xylol and passage through absolute ethanol and 95 per cent ethanol to physiological saline, the sections were stained for pneumococcal polysaccharide by use of a single layer technique, with fluorescein isothiocyanate conjugate of the crude globulin fraction of a rabbit anti-pneumococcus (type III) serum. The conjugate was applied over the section and left in a moist chamber at room temperature for 30 min., followed by a wash for 10 min. in physiological saline buffer (0·15 M-NaCl : 0·01 M-phosphate pH 7·0).

*Localization of injected diphtheria antitoxin and of antitoxin-containing cells in immunized animals.*—Frozen sections (3 $\mu$) were cut in a cryostat, thawed onto chromic acid cleaned slides, fixed with absolute methanol at room temperature (20°–22 °) for 15 min. and quickly washed in buffered saline. The sections were then exposed to a solution of diphtheria toxoid (500 Lf/ml.) for 30 min., washed in buffered saline for 5 min., exposed to horse diphtheria antitoxin labelled with fluorescein isothiocyanate and finally washed for 10 min. in physiological saline buffer. All fluorescent conjugates were absorbed twice with acetone lyophilized mouse liver powder. (100 mg. per ml.)

*Controls for staining procedures with fluorescent antibody conjugates*

*Pneumococcus polysaccharide.*—(i) Adjacent cryostat frozen sections were treated after fixation with a heterologous fluorescent antibody conjugate : fluorescein iso-thiocyanate conjugated rabbit anti-ovalbumin.

(ii) Adjacent cryostat frozen sections were treated with fluorescein conjugated anti-pneumococcus polysaccharide type III which had been absorbed with the calculated amount of homologous polysaccharide at 4° for 5 days followed by centrifugation to remove the antigen-antibody precipitate and checked to show no reaction with known polysaccharide containing sections.

*Diphtheria antitoxin.*—(i) Adjacent sections were treated after methanol fixation with fluorescein-isothiocyanate conjugated diphtheria antitoxin for 30 min., followed by a 10 min. wash in physiological saline-buffer. Such treatment would reveal diphtheria toxoid antigen and non-specifically stained structures.

(ii) Adjacent sections were treated after methanol fixation with a heterologous conjugate : fluorescein isothiocyanate rabbit anti-ovalbumin to reveal non-specifically stained structures.

*Fluorescence microscopy.*—The equipment previously described (White, 1960*a*), which employs ultraviolet radiation from a 20 amp. carbon arc source, filtered through 20 per cent. copper sulphate solution, was used. Visible radiation, was finally screened by a Corning 9840 2 mm. filter, with a Wratten 2B filter in the eyepiece.

## RESULTS

### *Effect of Direct Injection of Antigens into the Substance of the Thymus Gland*

In 6 guinea-pigs injected with multiple doses of $4 \times 10^7$ heat-killed typhoid-paratyphoid baccilli into the substance of the thymus, with an interval of 2–3

weeks and killed 5–9 days after the second series of injections, sections of the thymus showed two main histological changes. First, there were prominent confluent areas, often in close relation to the blood vessels or actually perivascular in situation, consisting of mature and immature plasma cells, and secondly, several lymphoid nodules with germinal centres (Fig. 1), apparently identical with those seen in other lymphoid organs, were present.

In the sections of 9 guinea-pigs injected with aluminium phosphate-precipitated diphtheria toxoid, large masses of amoeboid macrophages, with cytoplasm loaded with aluminium mineral salt particles were present in the medulla of the thymic lobules with adjacent areas of mature and immature plasma cells (Fig. 2). Under the fluorescence microscope these cytoplasmic particles fluoresced a bright greyish-blue (natural fluorescence). Segregated at the periphery of such masses, and often adjacent to or surrounding small blood vessels, and occupying the adventitia of these, were groups of antibody-containing mature and immature plasma cells (Figs. 3 and 4). The localization of such cells as revealed by the " sandwich technique ", which made use of fluorescein isothiocyanate-labelled diphtheria antitoxin after preliminary treatment of the section with dilute diphtheria toxoid, was checked by reference to adjacent frozen sections stained by Giemsa's method, and shown to correspond to groups of typical mature and immature plasma cells. The bright apple-green fluorescence was uniformly present throughout the cytoplasm or excluded a small juxta-nuclear area in the position of the Golgi element, and was also seen in some cells as irregular spots or rings within the dark oval of the nuclear area. It was apparent that the numbers of cells revealed as fluorescent antibody-containing cells were much less than the numbers of mature and immature plasma cells in corresponding areas of Giemsa-stained adjacent sections. Germinal centres were not seen in the thymuses of animals treated with aluminium phosphate-precipitated diphtheria toxoid. A surprising finding was bright fluorescence, indicating the presence of high concentrations of diphtheria antitoxin, in some of the Hassall's corpuscles. Control sections (see below) for the presence of homologous antigen or for the non-specificity of this reaction did reveal that these areas were to a slight extent non-specifically stained, but the difference between the test and control sections clearly indicated the presence of diphtheria antitoxin in high concentration at these sites.

Controls for the specificity of the staining reaction for diphtheria antitoxin were as follows :—First, sections stained by the same overall procedures as in the " sandwich technique " for diphtheria antitoxin, but omitting the treatment with dilute antigen (diphtheria toxoid) solution, resulted in no fluorescence in cells of plasma cellular morphology. Such control preparations showed very scanty cells with a cytoplasmic distribution of fluorescence in and among the masses of macrophages. From the granular nature of this fluorescence and the bi- or multi-lobed outlines of their nuclear shadows, these cells could be identified as polymorphonuclear leucocytes or eosinophils which had non-specifically localized the fluorescent globulin conjugate. The same controls showed that some of the cells in the centre of the medullary groups of macrophages had a faint apple-green cytoplasmic fluorescence, and that irregular green streaks and granules existed between such central cells. This was thought to indicate the presence of diphtheria toxoid antigen both within the cytoplasm of macrophages and extra-cellularly at these sites. The cytoplasm of surrounding macrophages fluoresced a bright greyish-blue owing to their content of aluminium mineral salt.

Secondly, sections stained with a heterologous fluorescein globulin conjugate (rabbit anti-ovalbumin) showed no localization to plasma cells, or to the central macrophages and sites of extracellular fluorescence seen in the above sections. However, the same scanty polymorphonuclear leucocytes or eosinophils as appeared in the above treatments also appeared in these control sections.

### *Vital Staining of the Normal and Injured Thymus*

For vital staining of the normal and injured thymus, 20 rats and 13 guinea-pigs were employed. The results were similar in both species. Marked staining of the macrophages in the connective tissue capsule and septae of the thymus was seen (Fig. 5) and in the rat, well marked staining of the sinus cells of the small lymph nodes on the external surface of the thymus was present (Fig. 6). In some animals no definite staining of cells in the medulla or cortex of the thymus could be found. In others occasional granules of dye could be picked up in macrophages, usually situated at the cortico-medullary junction or at the periphery of the thymic cortex. These results could not be explained by an absence of reticulo-endothelial cells from the gland, as metallic impregnation methods for microglia (Fig. 7) showed a dense plexus of cells in the medulla resembling those seen in other lymphoid organs and previously classified by one of us (Marshall, 1953), as " metalophil cells ". It would appear that these cells account for a large number of the cells in the medulla of the thymus usually classified as " epithelial reticular cells ". The cells of the Hassall's bodies however, remained unstained by this metallic impregnation method.

In the guinea-pig thymuses which had received a cautery incision, marked vital staining was seen in macrophages in the medulla of the thymus in the region

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EXPLANATION OF PLATES

FIG. 1.—Germinal centre in medulla of thymus following direct injection of TAB vaccine. H. and E. ×114.

FIG. 2.—Plasma cells in medulla of thymus at edge of area of PAPT deposit. H. and E. ×684.

FIG. 3.—Fluorescence micrograph : antibody-containing cells in thymus at margin of deposit of PAPT-containing macrophages. Diphtheria antitoxin fluorescein conjugate. ×114.

FIG. 4.—Fluorescence micrograph : antibody containing cells similar to those in Fig. 3. Diphtheria antitoxin-fluorescein conjugate ×456.

FIG. 5.—Vitally stained cells in connective tissue septae of rat thymus. Note unstained thymic tissue. Trypan blue. Neutral red counterstain. ×85.

FIG. 6.—Vitally stained cells in lymph node on surface of rat thymus. Note unstained adjacent thymic tissue. Trypan blue. Carmine counterstain. ×100.

FIG. 7.—Metalophil reticulo endothelial cells in medulla of rat thymus. Weil-Davenport method for microglia. ×135.

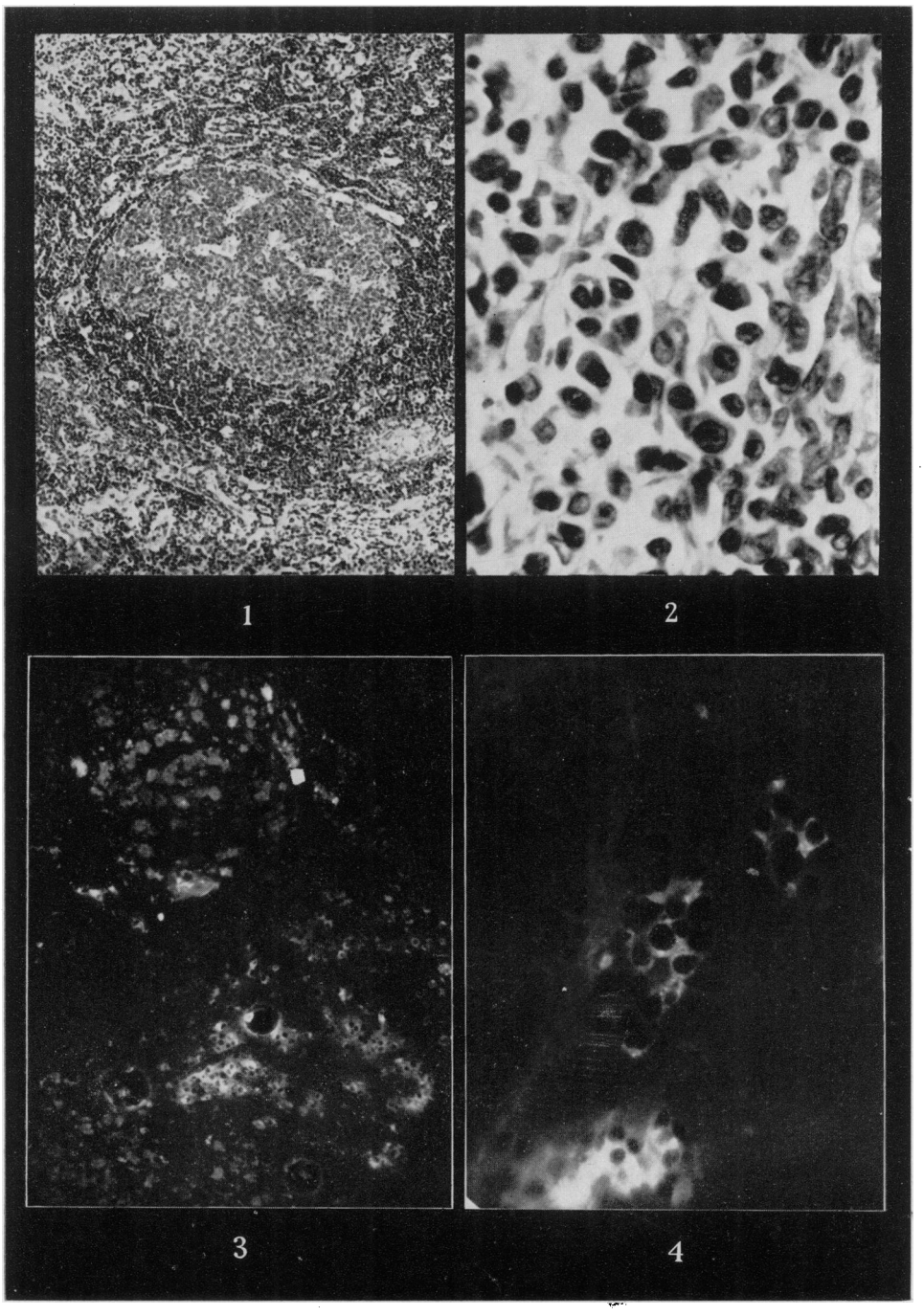
FIG. 8.—Marked vital staining of macrophages in medulla of thymus in region of cautery incision. Trypan blue. H. and E. ×315.

FIG. 9.—Fluorescence micrograph. Specific fluorescence denoting presence of pneumococcal polysaccharide in cells of rat thymic capsule, 48 hr. after injection.

FIG. 10.—Fluorescence micrograph. Marked specific fluorescence denoting presence of pneumococcal polysaccharide in lymph node on surface of rat thymus, 48 hr. after injection. Note fluorescent cells in adjacent thymic capsule and lack of staining in thymus. ×85.

FIG. 11.—Fluorescence micrograph. Pneumococcal polysaccharide in lumen of blood vessel in medulla of thymus. Non-specific fluorescence of Hassall's body at opposite side of figure. ×456.

FIG. 12.—Fluorescence micrograph. Numerous fluorescent polysaccharide-containing cells in region of cautery incision in guinea-pig thymus, 4 days after intravenous injection of pneumococcal polysaccharide. ×114.

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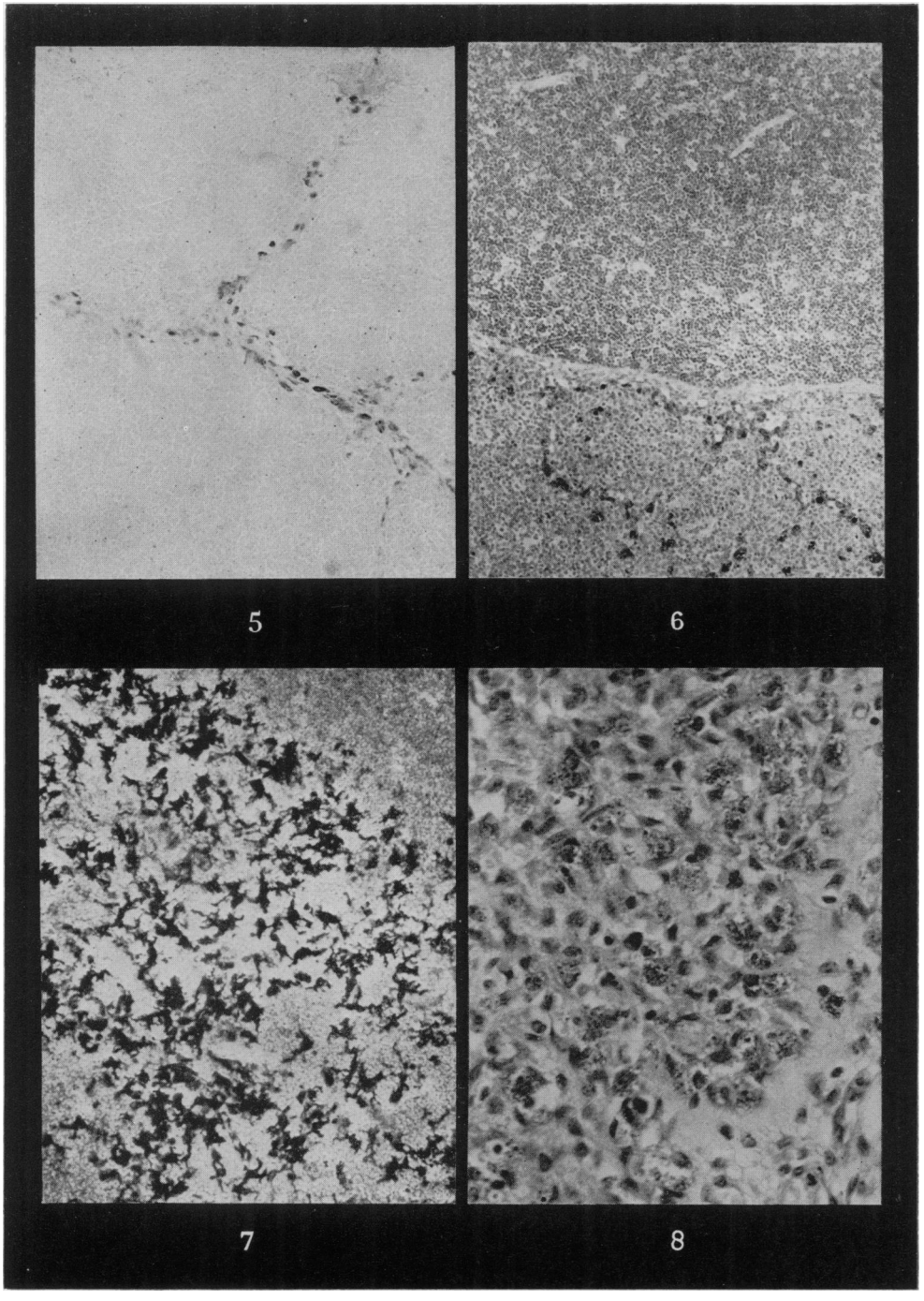

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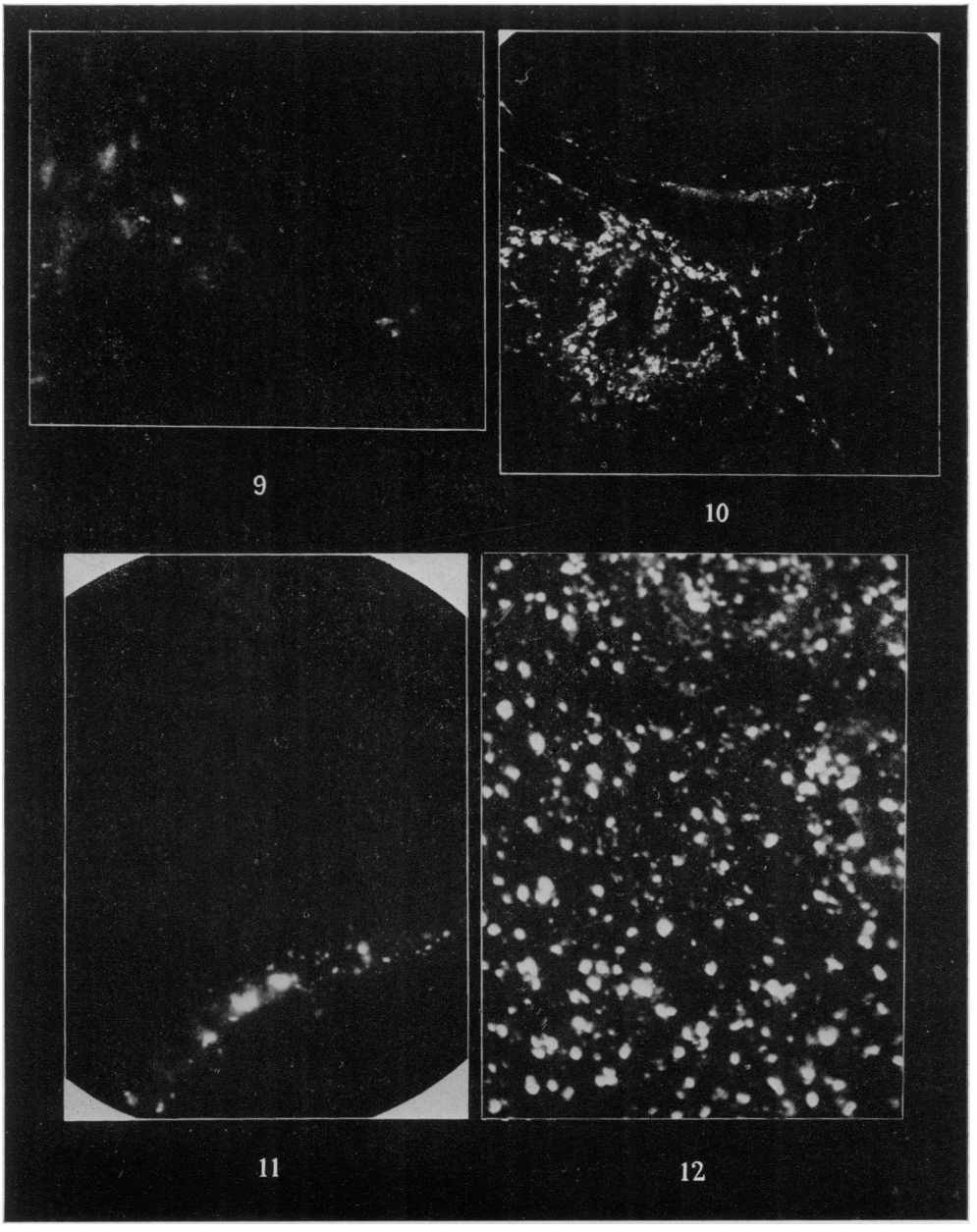

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of the incised area (Fig. 8). With metallic impregnation methods a large number of metalophil cells were seen in the same area, many showing transitional forms resembling those seen in the microglia of the brain following cerebral injury.

### *Distribution of Pneumococcal Polysaccharide in the Normal and Injured Thymus Following Intravenous Injection*

Seven guinea-pigs and 2 rats were employed. In the normal and injured thymus the distribution of injected polysaccharide closely followed that of vital dyes. Intense specific fluorescence was observed in cells in the capsule and interlobular connective tissue of the gland (Fig. 9). Marked fluorescence was also seen in the sinus-lining cells of the small lymph nodes attached to the thymic surface in the rat (Fig. 10) and in lymph nodes from other areas of the body. In animals killed 48 hr. after injection polysaccharide was present in considerable amounts in the lumen of blood vessels (Fig. 11). Some non-specific fluorescence was seen in the Hassall bodies (Fig. 11) and in the granules of eosinophil and neutrophil leucocytes in the medulla of the thymus. Only very slight specific fluorescence was observed in cells in the normal thymic cortex or medulla mainly in the region of small blood vessels, a result similar to that observed in the mouse thymus by Coons, Leduc, and Kaplan (1951) employing protein antigens. Following cautery injury in the guinea-pig large numbers of polysaccharide-containing fluorescent cells were observed in thymic tissue adjacent to the injured area (Fig. 12). Hassall bodies in the region of injury formed cyst-like structures containing cells with included fluorescent material.

## DISCUSSION

It has been shown that direct injection of antigens into the thymus produces the usual histological changes associated with antibody formation in other lymphoid organs in contrast to the complete lack of cellular reactivity observed by others following introduction of antigens into the blood stream. This failure of reaction to circulating antigen may be attributed either to a barrier against the entry of antigens into the thymus or to the lack of a suitable phagocytic mechanism to segregate it in the gland. The presence of large numbers of reticulo-endothelial cells, as shown by metallic impregnation methods for microglia, in the normal thymus and the rapid phagocytosis of both vital dye and antigenic material following local injury suggest that a barrier exists between the blood and the parenchyma of the thymus similar to that between the blood stream and the central nervous system ; admission of foreign material in quantity to the thymus occurring only following a change in local vascular permeability. The existence of such a barrier would therefore preclude any cellular reaction of the normal thymus to circulating antigen and any antibody formation in the organ.

These observations provide a basis for a possible interpretation of the nature of the lesion in the human thymus in myasthenia gravis. Between 60–70 per cent of cases of myasthenia (Castleman, 1955), show the presence of germinal centres in the medulla of the thymus, structures histologically identical with those seen in lymph nodes following repeated antigenic stimulation. The interpretation of this finding in the human thymus has been a matter of difficulty however, both owing to uncertainty about the significance of formation of germinal centres in other lymphoid tissue and also to the lack of production of a similar lesion in the

the thymus by experimental methods. There is now evidence however that production of germinal centres in lymph nodes may represent a reaction to antigenic stimuli which under certain circumstances results in demonstrable production of specific antibody or $\gamma$-globulin (White 1960*b*; Ortega and Mellors 1957), and it has been shown here that the thymus may also form germinal centres *ab initio* as a response to an antigen.

These observations suggest that the thymic lesion in such cases of myasthenia gravis may represent an immune response to an antigen. If the human thymus possesses a barrier to circulating antigen similar to that in animals such an immune response must arise primarily in the thymus gland. This hypothesis would indicate that the thymus is the primary agent in myasthenia gravis and not that a common disturbance gives rise both to the myasthenia and to the changes in the gland as suggested by Havard and Bodley Scott (1960), a conclusion which is also difficult to accept in view of the absence of reaction in other lymphoid organs in myasthenics (Castleman, 1955). It would further indicate that the basic disturbance in myasthenia gravis is immunological in nature, and is not related to the secretion of a hormonal agent by the gland. This view is compatible with both the presence of the characteristic " lymphorrhages " in the muscles of myasthenics a lesion which is also seen only in the muscles in rheumatoid arthritis; and with the predominantly female sex distribution, as often observed in auto-immune disease, of patients showing germinal centre formation in the thymus. Strauss *et al.* (1960) have recently claimed that the serum of patients with myasthenia gravis contains an antibody-like entity which reacts with homologous skeletal muscle and Simpson (1960) has suggested on clinical evidence that myasthenia gravis is an auto-immune disorder in which the thymus reacts to protein derived from the motor end plates. While our findings agree with the hypothesis of an auto-immune basis for this disease they are not compatible with the concept that the thymus reacts to circulating end plate protein or other muscle tissue antigen. On the other hand, the histological appearances in the myasthenic thymus may be compared with the histological reactions at the local injection site in the thymus of a small dose of a bacillary vaccine (TAB), or with those seen in the thyroid in Hashimoto's disease, which also appear to represent the response to a locally escaping antigen (White, 1957) and which prominently feature lymphoid nodules with germinal centres as a part of this immune cellular reaction. If the thymus gland is the primary source of the disease then either a common antigen must be shared by the thymus and motor nerve endings or according to the concepts of Burnet (1959), proliferation of a " forbidden clone " capable of forming antibody or reacting immunologically against nerve endings or muscle components must arise spontaneously in the gland. If the thymus is shielded by a blood-thymic barrier from contact with circulating proteins, it would appear a particularly favourable site for such clonal growth to take place, as contact with antigen is a mechanism postulated by Burnet for the destruction of such " forbidden clones " as are capable of immune responses against an individual's own tissues.

Further investigation is required of the finding of the presence of specific antibody, as reported here, and of gamma globulin (Gitlin, Landing and Whipple, 1953) in some Hassall's corpuscles. It is tempting to assume a common origin and function between the entodermal cells of such structures and the entodermal epithelial cells, which, according to Ackerman and Knouff (1959) and Burnet (1961) give rise to the cells of the germinal centres in the lymphoid nodules of the

bursa of Fabricius in the chicken. The latter shares with the thymus an epithelial origin from the gut and appears to resemble it ontogenetically by progress to a peak size at 4 months in the chicken and then by slow atrophy during the remainder of the bird's life.

SUMMARY

It has been shown that direct injection of antigens into the thymus of guinea-pigs induces the formation of germinal centres and plasma cells and the production of antibody.

The normal rat and guinea-pig thymus possesses a barrier against the entry of vital dyes or antigens from the blood stream into the gland which may be broken down by local trauma. The significance of these findings is discussed, especially in relation to the pathogenesis of myasthenia gravis.

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