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Immunological Responses following Injection of Antigens in Freund's Adjuvant into Thymus and Other Tissues

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Summary. Groups of young adult rats were inoculated with bovine serum albumin in complete Freund's adjuvant into one foot-pad, a cervical lymph node, the spleen, a forelimb muscle, the cavity of the mediastinum or directly into the thymus. Arthus responses and circulating antibodies occurred in decreasing intensity in the order listed, but marked delayed sensitization resulted in the foot-pad and lymph node groups only.

Groups of guinea-pigs were inoculated with egg albumin in complete Freund's adjuvant into a foot-pad, on the surface of the thymus or into the thymus. Delayed sensitization did not develop until the 17th day in the intrathymic group, and Arthus reactions and circulating antibodies were poor when compared with the other animals.

Allergic encephalomyelitis was found in all seven rats injected with spinal cord and adjuvant in one foot-pad and in five of seven animals injected into a cervical node, while a single minimal lesion was present in one of twelve animals inoculated into the thymus directly.

Plasma cells were seen in the thymus after injection of protein and adjuvant near the connective tissue of the capsule, the interlobular septa and blood vessels, whereas nervous tissue and adjuvant produced virtually no plasma-cell response.

INTRODUCTION

Several recent publications establish that the thymus gland, at least up to a few days after birth (Miller, 1961; Janković, Waksman and Arnason, 1962; Good, Dalmasso, Martinez, Archer, Pierce and Papermaster, 1962) and probably in adults as well (Miller, 1962), is essential to delayed sensitization, homograft rejection and certain types of antibody formation. It has been suggested that the thymus acts by producing lymphocytes which are precursors of the immunologically active cells found in other tissues, such as the spleen and lymph nodes (see Arnason, Janković and Waksman, 1962). A number of investigators in the past, including Askonas and White (1956) and Dixon, Weigle and Roberts (1957), have however found that the thymus cells *in situ* do not form antibody in animals immunized by a systemic route. The absence of response has been attributed by Marshall and White (1961) to the presence of a barrier between the blood and the thymus, as measured by the failure of vital dyes and pneumococcal polysaccharide injected systemically to penetrate the gland. These workers reported that when the barrier is transgressed by injection of killed typhoid and paratyphoid bacilli (TAB) or aluminium-precipitated

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diphtheria toxoid into the gland itself, antibody-forming plasma cells appear in the thymus and, in guinea-pigs injected with TAB, germinal centres may be formed in its medulla. These observations might lead to the inference that the cells of the thymus are immunologically competent.

The present paper reports an attempt to assess the immunological responsiveness of the thymus cells by another method. We injected either purified protein and adjuvant or spinal cord and adjuvant directly into the thymus and measured antibody production, the appearance of skin reactivity of the delayed type, and the development of allergic encephalomyelitis (EAE).

EXPERIMENTAL METHODS

Immunization Procedures

Sprague-Dawley rats*, 10–12 weeks old, weighing approximately 150 g. and 500–600 g. Hartley strain guinea-pigs† were used. The animals were anaesthetized with ether and the thymus injected with 0·05 ml. of complete Freund's adjuvant containing 250 μg. of bovine serum albumin (BSA)‡ for the rats, and 2 μg. of egg albumin (EA)§ for the guinea-pigs. The adjuvant mixture consisted of 10 volumes of BSA or EA solution, 1·5 volumes of Arlacel A,‖ 8·5 volumes of Bayol F¶ and killed *Mycobacterium tuberculosis*** at a final concentration of 3 mg./ml., emulsified to give a water-in-oil emulsion. Injection of the thymus in the guinea-pig is a simple operation since the gland is in the neck; in the rat however it was necessary to split the sternum for 3–4 mm. and separate the muscles of the neck. Often it was helpful to steady the upper end of the gland with a cotton-wool-tipped orange stick to prevent excessive movement of the mediastinum during respiration and to bring the gland into clearer view. At times there was a leak of injected material from the surface of the gland, and this had to be dabbed until the leakage stopped; at other times the injection was too deep. The wound was sutured in layers and the animal received 60,000 units of penicillin and 60 mg. of streptomycin intramuscularly. In additional groups of animals the same procedure was followed but the mixture was injected into the space over the surface of the thymus gland, i.e. into the cavity of the upper mediastinum. Further groups of animals received a similar quantity of adjuvant mixture injected into the left hind foot-pad (rats and guinea-pigs), cervical lymph node, forelimb muscle and spleen (rats only). During the injection of the spleen, globules of emulsion were often seen leaving the organ via the splenic vein.

Skin Tests

Rats were skin-tested 9–12 and 20–21 days after inoculation with 30 μg. of BSA in saline and old tuberculin†† 1/10. Guinea-pigs were tested on the 7th and 18th day after inoculation with 3 μg. of EA in saline. Arthus reactions were read at 3–4 hours and delayed skin reactions at 24 and 48 hours. Approximately one-quarter of the rats in each group received an intraperitoneal booster dose of 1 mg. of BSA on the 14th day. Since this

* Obtained from Charles River Breeding Laboratory, Brookline, Mass.
† Obtained from Tumblebrook Farm, Brant Lake, New York.
‡ Crystalline BSA, Pentex Inc., Kankaee, Illinois.
§ Crystalline EA, Armour & Co., Chicago 9, Illinois.
‖ Mannide monooleate, Atlas Powder Co., Wilmington, Delaware.
¶ A light mineral oil, Esso Standard Oil Co., Linden, New Jersey.
** Kindly provided by Dr. I. S. Danielson, Lederle Laboratories, Pearl River, New York.
†† Old Tuberculin, Human concentrated, Eli Lilly & Co., Indianapolis.

appeared to have no effect on their subsequent reactivity, data obtained from these animals were pooled with the remaining observations.

Antibody Titration

Rats were bled 23–24 days and guinea-pigs 19 days after inoculation. Sera were harvested, stored at −20°, and absorbed once with washed sheep red cell suspension (1 per cent) before use. Haemagglutinating antibody was measured by a modification of the microtitration technique of Takátsky (Janković *et al.*, 1962) using formalinized sheep red cells which had been treated with tannic acid and incubated with BSA or EA. Precipitating antibody in the rat sera was studied in Ouchterlony plates, with BSA at a concentration of 60 μg./ml. in the central well. The guinea-pig sera were studied in Oudin tubes with 50 per cent whole egg-white in saline, and in another series of tubes with crystalline egg albumin 1 mg./ml., layered above the agar–serum mixture.

Inoculation with Spinal Cord

Spinal cord was ground with Bayol F containing heat-killed *M. tuberculosis*, 3 mg./ml., until a uniform oily suspension containing 30–40 per cent spinal cord was obtained; 0·05 ml. of this mixture was injected into rats: in one group into the thymus, in another into a cervical node, and in a third into the left hind foot-pad.

Post-mortem Examination

Rats were sacrificed 23–27 days and guinea-pigs 19 days after inoculation. In the EAE experiments the animals were autopsied at 21 days or earlier if signs of disease were definite.

Paraffin sections which were 6–8 μ thick were stained with haematoxylin and eosin. In each animal which had received BSA, EA or spinal cord and adjuvant directly into the thymus the mediastinum was sectioned at two levels. Thus we could examine the thymus as well as a number of mediastinal lymph nodes. All injected cervical lymph nodes were sectioned. Only representative animals which had adjuvant mixture put on the surface of the thymus or injected into the spleen were examined. The lymphoid organs of animals injected into muscle or foot-pad were not studied. Sections of brain, cerebellum, brain stem and multiple levels of spinal cord were examined in all animals injected with spinal cord and adjuvant.

RESULTS

RATS

Delayed Skin Reactions

Normal rats tested with 30 μg. of BSA occasionally gave 24-hour skin reactions 5 mm. in diameter, hence readings up to this value were considered negative. As shown in Table 1, of animals injected in a foot-pad or a lymph node with BSA in adjuvant, the majority showed intense delayed-type sensitization by 10 days, waning at 20 days, whereas those given the same material into the spleen, muscle, or the upper mediastinum failed in general to become sensitized. Little or no sensitization was observed in animals receiving a direct intrathymic injection of antigen. The 48-hour skin reactions to BSA were closely parallel to the readings at 24 hours.

Tuberculin reactions (not shown in table) larger than 5 mm. were observed at 20 days in eight of fourteen animals which were injected into the foot-pad and three of thirteen animals which were injected in a cervical node. Of the remaining animals, only one in the muscle and one in the thymus groups developed positive responses.

TABLE 1

IMMUNOLOGICAL RESPONSES OF RATS GIVEN BSA AND ADJUVANT BY VARIOUS ROUTES

Pooled data of two experiments.

Site of inoculation	*No. of animals*	*Day of test*	*Arthus reaction*			*Delayed reaction (24 hours)*			*Haemagglutinating antibody 23rd to 24th day*		
			0–5 *mm.*	6–10 *mm.*	>10 *mm.*	0–5 *mm.*	6–10 *mm.*	>10 *mm.*	⩽1/32	1/64–1/256	⩾1/512
Thymus	23	9–12	22	1	0	21	2	0	12	9	2
		20–21	14	7	2	22	1	0			
Upper mediastinum	12	9–12	12	0	0	11	1	0	5	7	0
		20–21	10	2	0	10	2	0			
Muscle	6	9–12	5	1	0	6	0	0	1	5	0
		20–21	3	3	0	4	2	0			
Spleen	8	9–12	8	0	0	7	1	0	1	4	3
		20–21	2	3	3	5	3	0			
Lymph node	13	9–12	10	1	2	5	3	5	1	4	8
		20–21	0	10	3	2	11	0			
Foot-pad	14	9–12	3	10	1	3	1	10	0	5	9
		20–21	1	5	8	1	9	4			

Arthus Reactions

Almost all rats inoculated with BSA and adjuvant by the foot-pad route showed sensitivity at 10 days, which became intense by 20 days (Table 1). Animals injected in the spleen or lymph node showed little at 10 days but moderate to intense sensitivity at 20 days, while those injected in the muscle or upper mediastinum developed a lesser degree of sensitivity. In only one third of the animals given antigen directly into the thymus was moderate sensitization observed at 20 days.

Antibody Formation

Haemagglutinating antibody, measured at 23–24 days, was closely correlated with Arthus reactivity (Table 1), the titres being high in foot-pad and lymph node groups, moderate to high in the spleen group, and low to moderate in the group inoculated in the muscle and upper mediastinum. Of the animals receiving intrathymic antigen, twelve failed to show significant production of antibody (1/32), nine had antibody titres greater than 1/32 and positive Arthus reactions, and two with antibody titres of 1/64 and 1/128 had no Arthus responses.

Precipitin analysis in Ouchterlony plates gave results identical with the findings by haemagglutination, except for a single serum (from an animal immunized by placing antigen on the thymus), which gave a faint line in dilution of 1/4 though negative by haemagglutination.

Production of Allergic Encephalomyelitis

Six of seven animals injected into the foot-pad with spinal cord adjuvant and five of seven injected into a cervical node developed typical allergic encephalomyelitis, with characteristic onset 8–12 days after inoculation (Table 2). In all these animals as well as in the seventh animal of the foot-pad group, characteristic histological lesions were found

TABLE 2

ALLERGIC ENCEPHALOMYELITIS IN RATS INOCULATED BY VARIOUS ROUTES

Pooled data of two experiments.

Site of inoculation	*No. of animals*	*Clinical disease*	*Histological lesions**	*Onset (days after inoculation)*	*Histological grading*
Thymus	12	0	1	—	+
Cervical node	7	5	5	8–12	+ + − + + +
Foot-pad	7	6	7	9–11†	+ − + + +

* All animals autopsied after onset of clinical disease or at 21 days.
† One rat had onset of disease at 16 days.

in the white matter of the cerebellum, the brain-stem and the spinal cord. It was our impression that the lesions were more severe in the animals injected via the cervical node, although the result is not significant in such a small series of animals. By contrast none of the animals inoculated into the thymus developed clinical disease, and only one showed a minimal lesion near the fourth ventricle.

GUINEA-PIGS

Delayed Skin Reactions

Animals inoculated with EA and adjuvant via a foot-pad or by placing the inoculation mixture on the thymus developed intense delayed reactivity by 7 days (Table 3). Of those receiving antigen directly in the thymus, the majority failed to react till 17 days.

TABLE 3

IMMUNOLOGICAL RESPONSES IN GUINEA-PIGS GIVEN EGG ALBUMIN AND ADJUVANT BY VARIOUS ROUTES

Site of inoculation	*No. of animals*	*Day of test*	*Arthus reaction*			*Delayed reaction (24 hours)*			*Haemagglutinating antibody 18th day*		
			0–5 *mm.*	6–10 *mm.*	>10 *mm.*	0–5 *mm.*	6–10 *mm.*	>10 *mm.*	0–1/8	1/16–1/32	1/64–1/256
Thymus	9	7	9	0	0	7	0	2	6	2	1
		17	3	1	5	1	1	7			
On surface of thymus	6	7	6	0	0	1	0	5	1	5	0
		17	0	1	5	0	0	6			
Foot-pad	5	7	3	2	0	0	0	5	0	1	4
		17	0	0	5	0	0	5			

Arthus Reactions

Arthus reactivity was not observed at 7 days but was present at a high level by 17 days in both foot-pad and 'on thymus' groups (Table 3). Two-thirds of those receiving intrathymic antigen also reacted at this time.

Antibody Formation

Haemagglutinating antibody against EA, measured at 18 days, was high in the foot-pad, moderate in the 'on thymus' group, and low in the thymus groups (Table 3). Dilutions of sera equal to or greater than 1/32 gave a single line in gel diffusion (Oudin) against whole egg white. With crystalline EA a line was only obtained with sera diluted 1/64 or more.

HISTOLOGICAL FINDINGS

Rat Thymus Injected with BSA and Adjuvant

The thymus, examined 23–24 days after local inoculation with protein and adjuvant, showed a patchy response, frequently with loss of normal architecture. Lesions often seemed to be confined by interlobular septa, but in many sections the changes extended beyond these. Some intact lobules always remained in damaged lobes.

The histological appearance consisted of dispersed oil droplets ('vacuoles') which were sometimes surrounded by polymorphonuclear leucocytes and fibrous connective tissue, but even immediately adjacent to oil vacuoles, there were relatively few areas of epithelioid cells. However, on the surface of the thymus and in the adjacent mediastinal connective tissue epithelioid cell masses were often extensive.

Plasma cell formation was very variable. When scattered vacuoles were within a lobule and the latter maintained its normal architecture, no plasma cells were present or isolated cells occurred. Where the injected material extended outside the gland, plasma cells were abundant. Nests or islands of these cells were particularly prominent in relation to blood vessels (Fig. 1a). Where thymus lobules were completely replaced by connective tissue, nests or islands of plasma cells were found also in relation to vessels. In some instances, there appeared to be extensive plasma cell formation along a line corresponding to the original connective tissue capsule of a lobule. Nodular masses of lymphocytes (follicles) or formations resembling germinal centres were not seen.

Seventy-six lymph nodes were seen in the coronal sections of mediastinum from the twenty-three animals which received intrathymic injection. In only three of these did we find oil vacuoles or epithelioid cells, i.e. evidence of penetration of the injected adjuvant mixture into the node. These animals had haemagglutination titres of 1/16, 1/32 and 1/28.

Guinea-Pig Thymus Injected with EA and Adjuvant

The response of the guinea-pig thymus differed in no way from that of the rat except that the granulation tissue in the former was more richly vascularized and many oil globules were found adjacent to the vessels.

Rat Thymus Injected with Spinal Cord and Adjuvant

The cellular response was remarkably different from that induced in the thymus by protein and adjuvant. While many oil vacuoles were seen in the substance of the thymus, there was no response by the thymic tissue in most instances (Figs. 2 and 3). Normal cortex and medulla could be seen immediately adjacent to both large and small vacuoles. In a few cases, there was a thin rim of epithelioid cells partly or completely surrounding a vacuole, often resembling flattened epithelium. When there were more than one or two layers of cells, the outer ones were clearly epithelioid in appearance. Very few plasma cells were seen in the thymus. However, where oil vacuoles were found in the capsular connective tissue, scattered plasma cells were present.

Rat Lymph Node Injected with Protein or Spinal Cord and Adjuvant

The reaction differed from that of the thymus in a number of particulars. The oil globules seemed to be more widely disseminated and there was a more intense epithelioid cell reaction in the node than in the thymus. However, many oil vacuoles had little reaction around them. The injected node as a whole was enlarged, and with either type of antigen, there were large lymphocytic masses in the cortex and masses of plasma cells in the medulla.

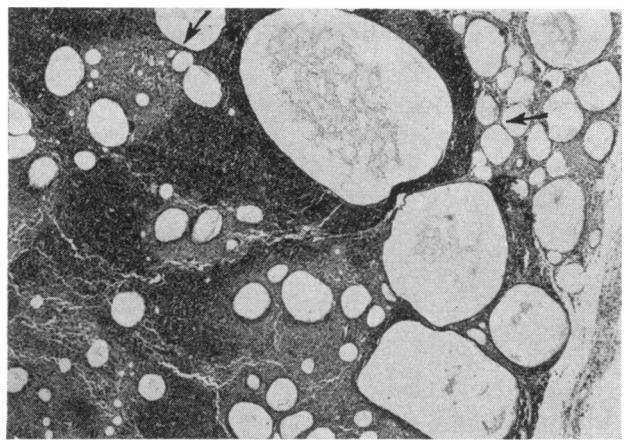

FIG. 2. Rat inoculated in thymus with spinal cord and adjuvant. Disseminated oil vacuoles within thymus and on surface. Arrows indicate fields shown in Fig. 3. ×36.

Rat Spleen Injected with Protein or Spinal Cord and Adjuvant

The response was widespread, often involving one third of the transverse section of the spleen, and was similar in every way to that of the lymph node. Plasma cells were present in large numbers, and were clearly related to blood vessels. There was no relative increase in the white pulp.

Lesions of Central Nervous Tissue in Rats Injected with Spinal Cord and Adjuvant

The lesions in sections of brain and spinal cord, were comparable with those described by a number of authors, being most prominent in the medulla and cerebellum, less frequent in the spinal cord and still less in the cerebral cortex. They consisted of perivascular cuffs of lymphocytes and histiocytes and paravascular infiltrates of the same cells, predominantly in the white matter, and a variable degree of meningitis with subpial mononuclear cell infiltration.

DISCUSSION

In view of evidence linking the thymus as an organ to immunological function (Miller, Marshall and White, 1962), the capacity of thymus lymphocytes to react immunologically is of some interest. In a recent study, Marshall and White (1961) injected the thymus of guinea-pigs with TAB vaccine and aluminium-precipitated diphtheria toxoid. With the former antigen germinal centres were formed in the thymus gland and in both cases

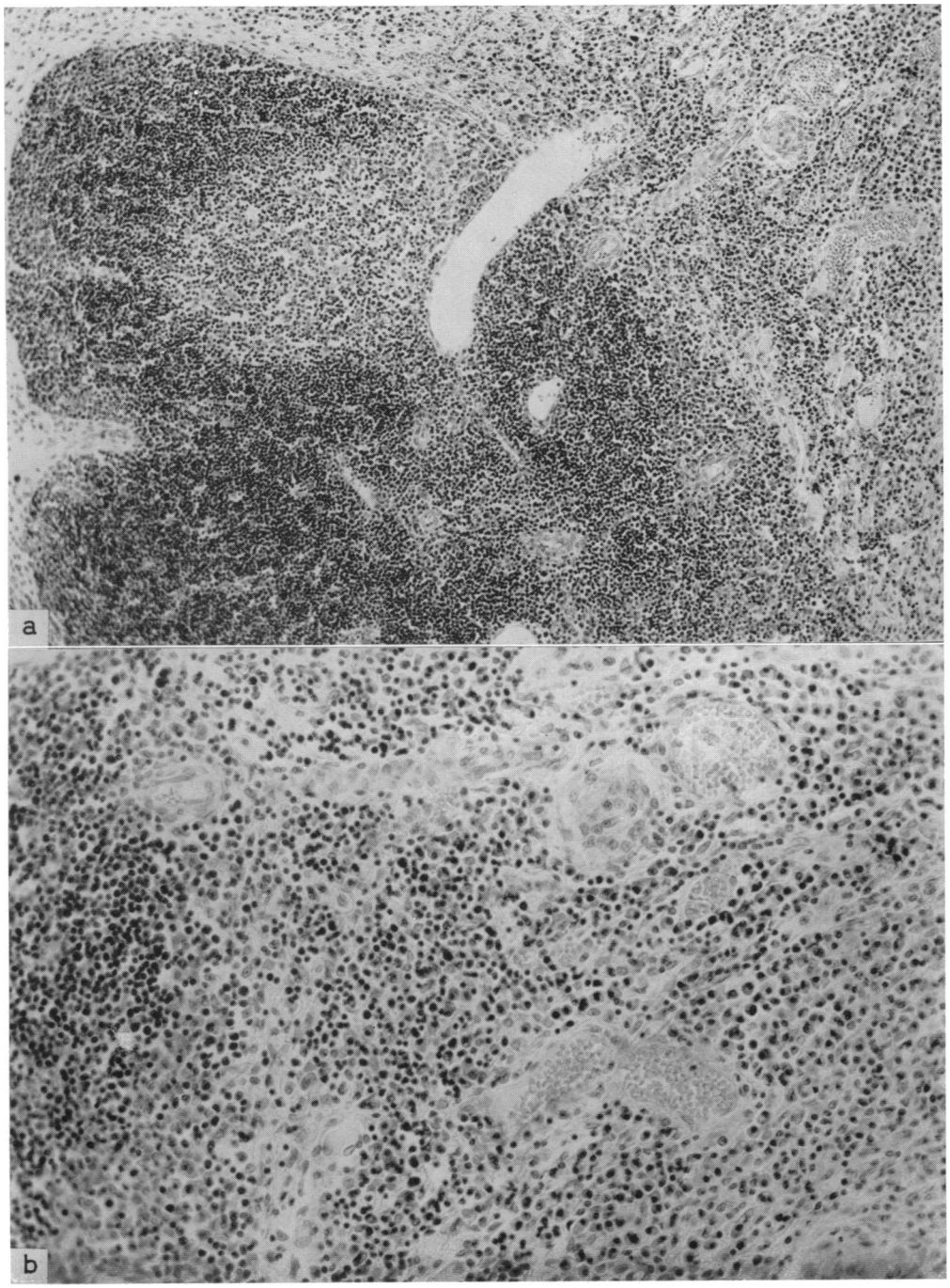

FIG. 1. Rat inoculated in thymus with BSA and adjuvant. (a) On the left normal thymus cortex and medulla are visible. The middle lower zone contains alteration of a rim of thymus cortex adjacent to the massive inflammatory zone on the right. ×120. (b) Higher magnification of inflammatory zone shows that the infiltrate contains many plasma cells, particularly localized about dilated vessels. Very few plasma cells are present in altered thymus cortex. ×216.

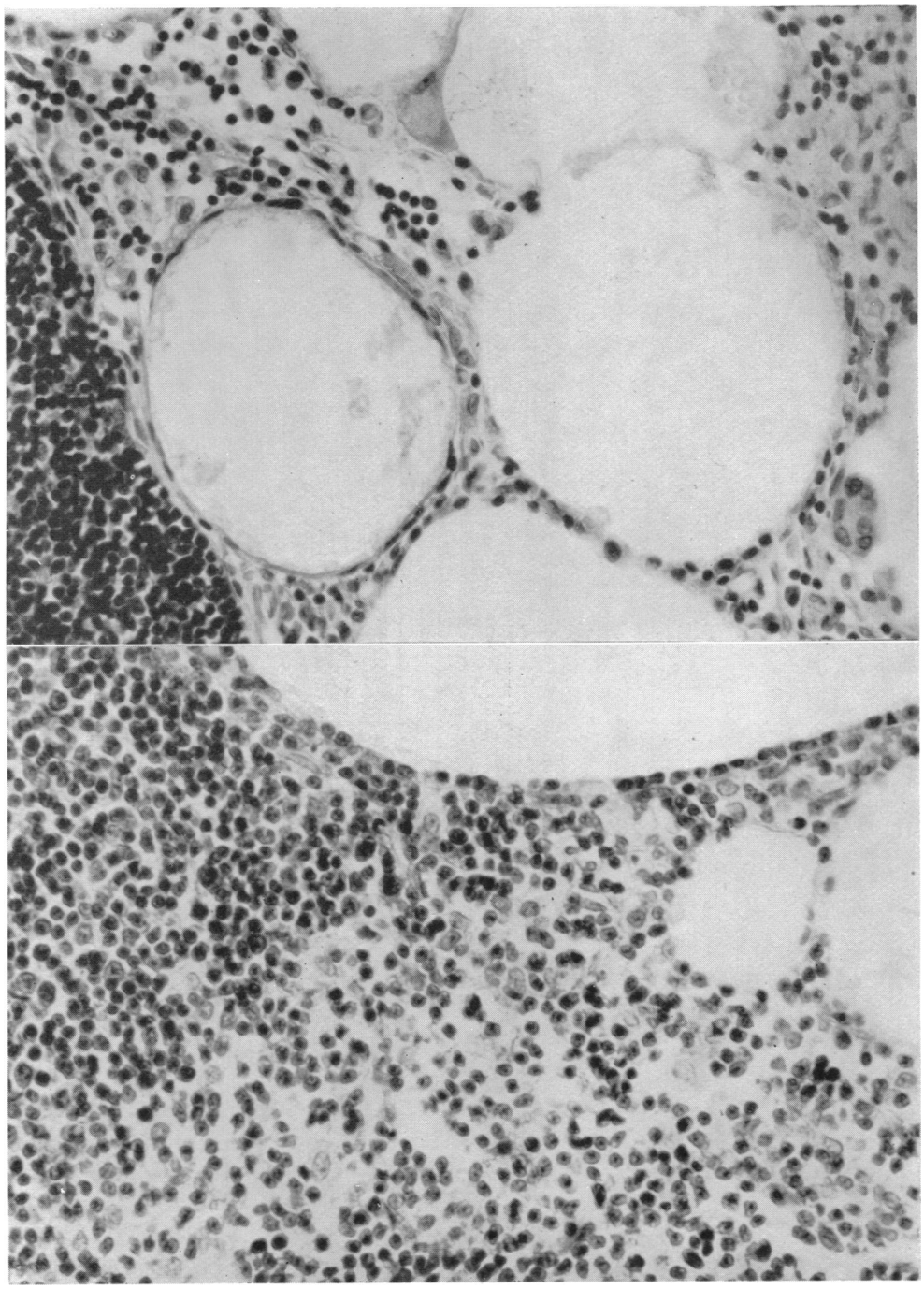

FIG. 3. Higher power views of thymus illustrated in Fig. 2. Upper field shows mild inflammatory response near oil vacuoles on surface of thymus with giant cells and occasional plasma cells. Lower field shows essentially normal thymus cortex and medulla immediately adjacent to several vacuoles; no plasma cells are seen. ×450.

antibody-forming plasma cells were shown by the fluorescent antibody method to be present, usually adjacent to blood vessels.

While different types of cells may be concerned in different immune responses, the only one about which there is general agreement at present is the plasma cell (Fagraeus, 1948; Leduc, Coons and Connolly, 1955). Therefore in our histological study of the thymus, we concentrated on two points: identification of the distribution of the injected antigen plus adjuvant by the presence of oil vacuoles, and assessment of the intensity of the plasma cell response. We made the assumption that these provided the best histological evidence for the distribution and intensity of the local immune response.

Our observations appear to confirm Marshall and White's finding that some plasma cells appear in the thymus injected directly with antigen. We did not, however, find germinal centres. Plasma cells were numerous in extrathymic connective tissue, into which the adjuvant mixtures presumably leaked, and also in and under the capsule of the thymus gland. However, in the thymus itself, when the structure of the organ was preserved, they were very infrequent. When a lobule was largely replaced by epithelioid cells and fibrous connective tissue, they were sometimes numerous, always in the form of nests, usually clearly related to vessels. The scarcity of plasma cells contrasted with the proliferation of these cells when a lymph node was injected. In this respect the response of the thymus was comparable to that of any non-lymphoid tissue injected with adjuvant mixtures, e.g. the toe-pad (White, Coons and Connolly, 1955) although these authors found few plasma cells in the draining node also. As in other tissues, one cannot distinguish between the possibility (a) that the plasma cells observed arise from competent precursor cells present locally or (b) that they arise from haematogenous precursors. In either event, they were always found in close relation to blood vessels or vascular connective tissue. In rats injected with nervous tissue and adjuvant, in whom sensitization of the delayed (cellular) type may be expected to predominate, almost no intrathymic plasma cells were found.

Our best method of judging the functional significance of these plasma cells was the measurement of both antibody formation and cellular sensitization. By this criterion, injection into the thymus was an extremely inefficient method of inducing an immune response. In the formation of haemagglutinating and precipitating antibody, the development of Arthus and delayed skin reactivity, the response obtained by the thymic route was inferior compared with that obtained by injecting adjuvant mixture into muscle or the mediastinal cavity and far inferior to the response after injection directly into a lymph node or a foot-pad. What little response there was presumably depended on the cellular reaction in and about the thymus, of which the plasma cells provided evidence. There was no sign of adjuvant vacuoles in or activation of adjacent mediastinal lymph nodes.

The most striking finding was the ease of production of EAE following the injection of spinal cord and adjuvant into a foot-pad or a lymph node compared with nearly complete failure to produce disease when the intrathymic route was utilized.

These observations carry the implication that thymus cells *in situ* are not immunologically competent or that only a small proportion of these cells may be capable of an immune response. This conclusion agrees with earlier findings in a number of different types of experimental situation (see discussion by Arnason *et al.*, 1962). Thus, rat thymocytes were found to have a very feeble ability to produce runt disease even in high doses (Billingham, Defendi, Silvers and Steinmuller, 1962); and the production of secondary disease in lethally irradiated mice required thirty times as many homologous thymus as lymph node

cells (Vos, DeVries, Collenteur and Van Bekkum, 1959). If thymocytes are nevertheless the precursors of immunologically active cells in other tissues, some explanation must be found to account for their feeble immunological capacity *in situ*.

The possibilities to be considered are the following: that the intrathymic environment prevents an immune response from taking place, that it lacks elements or possibly other cells essential to an immune response, that thymus lymphocytes are not in fact the precursors of immunologically reactive cells, or finally that they are in some way biochemically inadequate or 'immature'. Since thymocytes are far less effective in producing runt disease than an equal number of lymphocytes, it would appear that most of the thymocytes are immunologically deficient in this respect, and that it is the fault of the cells themselves rather than an effect of the intrathymic environment upon them. It is possible that they must undergo some 'maturation' process, either just before leaving the thymus, in the blood stream, or on arrival in another lymphoid organ.

The recent report of Svet-Moldavsky and Raffkina (1963), that plasma cells appear in the thymus glands of rats inoculated with adjuvant mixtures in the foot-pads and subcutaneously and germinal centres in those of monkeys given brain and adjuvant, would appear to conflict with our data and conclusions. One may conjecture that the changes they observed are a consequence of the widespread embolization with droplets of adjuvant commonly seen in animals given adjuvant mixtures by any route. Alternatively, the plasma cell formation may be part of the widespread formation of those cells, seen in a variety of tissues in heavily immunized animals (Bjørneboe and Gormsen, 1943).

The immunological responses obtained from animals injected into the spleen were better than those inoculated into the thymus, but not as good as the responses from animals immunized into a lymph node or the skin. This is interesting since the spleen undoubtedly contains immunologically competent cells. These results agree with those of Oakley, Batty and Warrack (1951) who failed to find specific local antibody production in the spleen when this site was injected directly. Their animals were primarily immunized via the skin and a secondary antibody response was obtained locally after a single injection into the skin, but not after injection into the spleen. This may imply that it is the mode of 'presentation' of the antigen to the immunologically reactive cell that plays an important role in the sensitization mechanism.

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