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The Formation of Immunoglobulins by Human Tissues *in vitro*

III. SPLEEN, LYMPH NODES, BONE MARROW AND THYMUS

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Summary. The site of immunoglobulin formation in human tissues was studied with three techniques: incubation of tissue fragments with radioactive amino acids followed by autoradiographic analysis of the synthesized immunoglobulins, immunofluorescent staining of tissue sections and cell suspensions, and morphological examination.

These studies showed that the spleen, lymph nodes and bone marrow synthesize IgG, IgA and IgM. In agammaglobulinaemia, one bone marrow sample showed no immunoglobulin synthesis, the other sample synthesized a trace of IgG. Immunofluorescent staining has demonstrated that in the spleen and lymph nodes plasma cells and large and medium-sized lymphocytes were positive for IgG, IgA or IgM; small lymphocytes were only positive for IgM. In bone-marrow samples, however, only plasma cells were positive for immunoglobulins. It is discussed whether in the bone marrow the cells that synthesize immunoglobulins do not originate in this organ, but derive from other lymphopoietic organs.

The normal thymus showed a different pattern because it synthesized only IgG and IgA. The plasma cells and medium-sized lymphocytes, which synthesize immunoglobulins, were localized predominantly in the interstitial connective tissue and ocasionally in the medulla, both near blood vessels. In all likelihood these cells did not originate in the thymus, but were trapped in this organ from the circulation. In autoimmune diseases, however, the thymus showed IgM-positive germinal centres and plasma cells and synthesized IgG, IgA and also IgM.

INTRODUCTION

The main sites of immunoglobulin formation are the plasma cells and the lymphoid cells of the spleen, lymph nodes and bone marrow (see review by Thorbecke and Benacerraf, 1962). This conclusion is based on studies carried out for the most part with animal tissues and performed prior to the introduction by Hochwald, Thorbecke and Asofsky (1961) of a technique which enables one to investigate the synthesis of the individual immunoglobulins. This method, applied to primate tissues, demonstrated the synthesis of different immunoglobulins by the lymph nodes, spleen, bone marrow and lymphoid tissues of the ileum and appendix (Asofsky and Thorbecke, 1961; Thorbecke, Asofsky, Hochwald and Jacobson, 1964).

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The cellular localization of immunoglobulin synthesis in the reticulo-endothelial organs of animals and man has been studied by immunofluorescent staining. The results indicate that cells of the plasma-cell series and the lymphoid cells in the germinal centres contain immunoglobulins (Coons, Leduc and Conolly, 1955; Leduc, Coons and Conolly, 1955; White, Coons and Conolly, 1955; Ortega and Mellors, 1957; White, 1960; Vazquez, 1961; Baney, Vazquez and Dixon, 1962; Balfour, Cooper and Alpen, 1965). This has also been established recently for the individual immunoglobulins (Cruchaud, Rosen, Craig, Janeway and Gitlin, 1962; Mellors and Korngold, 1963; Chiappino and Pernis, 1964).

In the present study, the synthesis of the different immunoglobulins by various organs of the reticulo-endothelial system was systematically investigated with a combination of three techniques: incubation of tissues with radioactive amino acids and subsequently analysis of the synthesized immunoglobulins by autoradiography of the immunoelectrophoretic pattern; direct immunofluorescent staining; and morphological examination of tissue sections and cell suspensions.

MATERIALS AND METHODS

For this study, 'normal' tissue is defined as tissue deriving from patients not suffering from a manifest infectious or autoimmune disease, who underwent surgery for non-inflammatory lesions, and in whom biopsy material did not reveal an increase of lymphoid elements, plasma cells or germinal centres, or any neoplastic infiltration.

Spleen samples were obtained from three patients who underwent splenectomy for hereditary spherocytosis and from one patient with hypersplenism of unknown origin combined with thrombocytopenia. The culture weights ranged from 69 to 223 mg.

Mesenteric and retroperitoneal lymph nodes were obtained from eight patients during abdominal surgery. The culture weights ranged from 71 to 197 mg.

Bone-marrow samples were obtained from iliac-crest puncture of five patients during neurosurgery for disc prolapse, from a sternum biopsy of a patient who had been treated successfully for an iron-deficiency anaemia, and from a rib of a patient undergoing pneumonectomy for bronchial carcinoma. The culture weights ranged from 47 to 167 mg.

Bone marrow samples from two patients suffering from idiopathic agammaglobulinaemia were also investigated. Patient B, a female of 25 years, suffered from a congenital, sporadic, non-sex-linked form of agammaglobulinaemia; the other patient (K), a male of 42 years, had an acquired form of primary hypogammaglobulinaemia. The culture weights were 110 and 96 mg respectively. In the double-diffusion test in agar gel only trace amounts of the three immunoglobulins were demonstrated in both sera.

Thymus biopsy samples were obtained during cardiac surgery from fifteen patients with a congenital heart disease and from two cases with an acquired mitral valvular lesion. The age of the patients was between 3 months and 42 years. The culture weights ranged from 72 to 373 mg.

Two thymus biopsy samples from patients with an autoimmune disease were studied. One sample was obtained from a 47-year-old female patient who had suffered for 7 years from systemic lupus erythematosus and who underwent cardiac surgery for a congenital heart disease; during that period she was clinically in remission and received no treatment. The second sample was from a woman of 50 years who underwent thymectomy after having suffered for 3 years from a severe form of myasthenia gravis which had not responded to medical treatment up to the time of sampling.

Biopsy samples from the lung (four), *thyroid gland* (three) *and liver* (three) were obtained during surgery to investigate the capability of the lymphoid elements in these organs to synthesize immunoglobulins. The liver biopsies were taken from the same patients with hereditary spherocytosis whose spleens were cultured.

Technical details of the methods applied have been described comprehensively in previous papers (van Furth, 1964; van Furth, Schuit and Hijmans, 1966a). The incubation studies were performed according to the method of Hochwald *et al.* (1961) with minor modifications. The direct immunofluorescent staining and the histological and cytological studies were done in frozen sections, cell suspensions and imprints.

RESULTS

SPLEEN

The *morphological study* of the spleen revealed a normal distribution of the white pulp which contained lymphoid cells with a rim of weakly-pyroninophylic cytoplasm. At the periphery of the white pulp and scattered in the red pulp several pyroninophylic plasma cells were seen (Fig. 1).

TABLE 1

COMBINED RESULTS OF AUTORADIOGRAPHS OF HUMAN SPLEEN, LYMPH NODE, BONE MARROW AND THYMUS CULTURES

Organs	Autoradiography*		
	IgG	IgA	IgM
Spleen	++	++	+
Lymph node	++	+	+
Bone marrow	+++	++	+
Thymus	++	(+)	−

* The intensity of the autoradiographic image is graded from: − = negative; (+) = just visible; + = clearly visible; to ++++ = very dark.

The *autoradiographs* of the four culture fluids demonstrated a distinct synthesis of IgG, IgA and IgM (Fig. 2, Table 1).

The *immunofluorescent staining* performed in frozen sections showed clusters of weakly-positive lymphoid cells in the white pulp region and scattered brightly-fluorescent plasma cells positive for IgG; the IgM-positive cells had the same appearance and localization (Fig. 1). Staining of the cell suspensions for IgG showed many brightly-fluorescent plasma cells, medium-sized lymphocytes, and a few larger cells. The IgA-positive cells in general showed the same morphology but were less numerous and the intensity of the fluorescence was lower. The immunofluorescent staining of the suspensions for IgM showed numerous brilliantly-fluorescent plasma cells and medium-sized cells, and an occasional large positive cell. In addition, a large number of small lymphocytes with a small rim of weakly-fluorescent cytoplasm positive only for IgM were observed (Fig. 3).

LYMPH NODES

The *morphological study* of the lymph nodes showed a normal structure with a cortex containing follicular centres and a clearly distinguishable medulla with a small number of plasma cells.

The *autoradiographs* of all culture fluids showed distinct synthesis of IgG and a small amount of IgA. IgM was synthesized in only six of the eight samples (Table 1). There was no correlation between these results and the culture weights.

The *immunofluorescent staining* of cell suspensions for IgG and IgA showed many brilliantly-fluorescent plasma cells, and medium-sized lymphocytes with a distinct rim of cytoplasm. The IgM-positive cells had the same appearance, but in addition many small lymphocytes with only a weakly-fluorescent rim of cytoplasm were observed.

BONE MARROW

The *morphology* and differentiation of the erythropoietic, leukopoietic and thrombocytopoietic cells of the normal bone-marrow samples were in the normal range, the number of lymphoid cells and plasma cells showed no increase, and no lymph follicles were observed.

The *autoradiographs* of the seven culture fluids showed the synthesis of a considerable amount of IgG and lesser amounts of IgA and IgM (Fig. 4, Table 1).

The *immunofluorescent staining* of all the bone marrow samples showed that the staining of IgG, IgA and IgM was positive exclusively in cells of the plasma-cell series; however, the lymphocytes failed to show fluorescence.

The samples obtained from the two patients suffering from an *agammaglobulinaemia* did not show plasma cells. The autoradiography of one sample (B) was consistently negative and the autoradiograph of the other showed a trace of IgG. The immunofluorescent staining revealed no positive cells.

THYMUS

The *histological* sections of all samples show, regardless of the age of the patient, a peripheral cortical area consisting mainly of densely-packed lymphocytes and a medulla containing a relatively large number of reticular cells, medium-sized and small lymphocytes, and Hassall's bodies. Germinal centres were not found in any of the thymus sections. The thymus structures in the older individuals had been partly replaced by fat tissue (Fig. 5). A considerable number of distinctly pyroninophylic cells with the morphological aspect of plasma cells were seen. These cells were predominantly located in the more vascular area of the connective tissue, which surrounded each lobule and penetrated deeply between them; but a small number was present in the medulla and an occasional cell was found in the cortex.

The *autoradiographs* of the culture fluids demonstrate in all seventeen samples the synthesis of a distinct amount of IgG and in ten of these samples a very small amount of IgA. The results for IgM were consistently negative except in one sample from a child of 9 months in which a trace of IgM was found (Fig. 6, Table 1). Two thymus cultures made with the routine medium omitting ovalbumin, to study whether ovalbumin would act as antigenic stimulus *in vitro*, also showed the synthesis of IgG and IgA.

Immunofluorescent staining showed a small number of plasma cells clearly positive for IgG and IgA, which were localized in the connective tissue surrounding the lobules and occasionally in the medulla (Fig. 5). In the cortex no IgG-positive cells were seen. Scattered in the sections a few lymphocytes were faintly positive for IgM. All cell suspensions showed several strongly IgG-positive plasma cells and clearly-positive medium-sized lymphocytes; the IgA-positive cells had the same appearance but were fewer in number. In

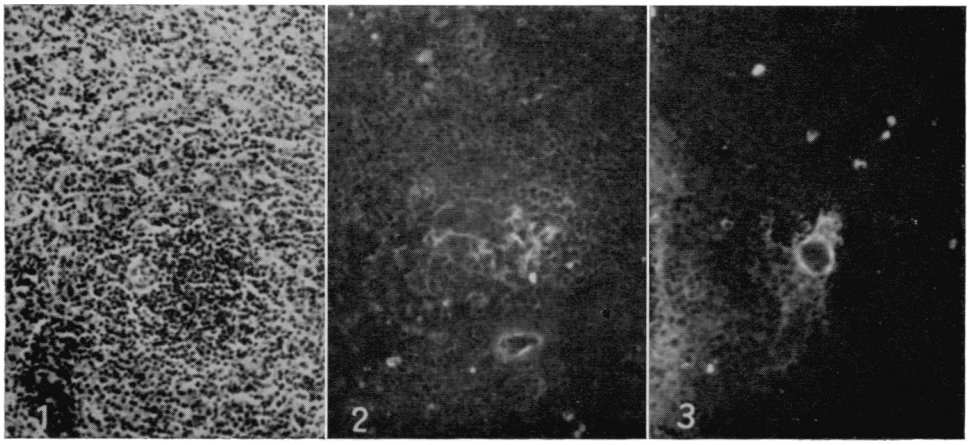

FIG. 1. Spleen. (1) White pulp H & E stained; (2) weak IgM-positive fluorescence in clusters of lymphoid cells of the white pulp and bright fluorescence in scattered plasma cells; (3) scattered brightly-fluorescent plasma cells positive for IgG. × 100

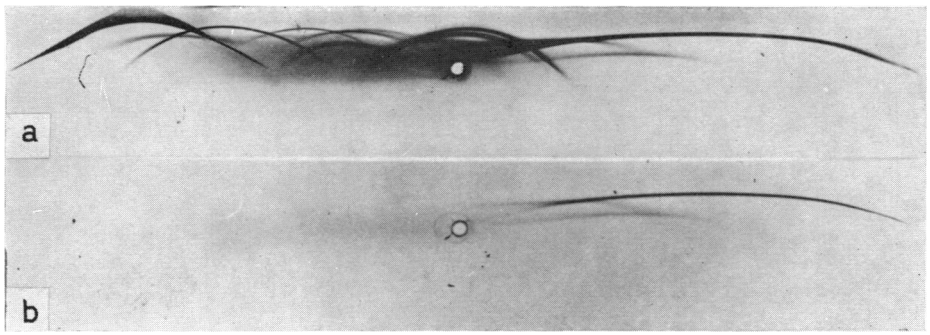

FIG. 2. Spleen. (a) Immunoelectrophoretic pattern of carrier serum and culture fluid developed with horse anti-human antiserum; (b) autoradiograph with labelling of IgG, IgA and IgM lines.

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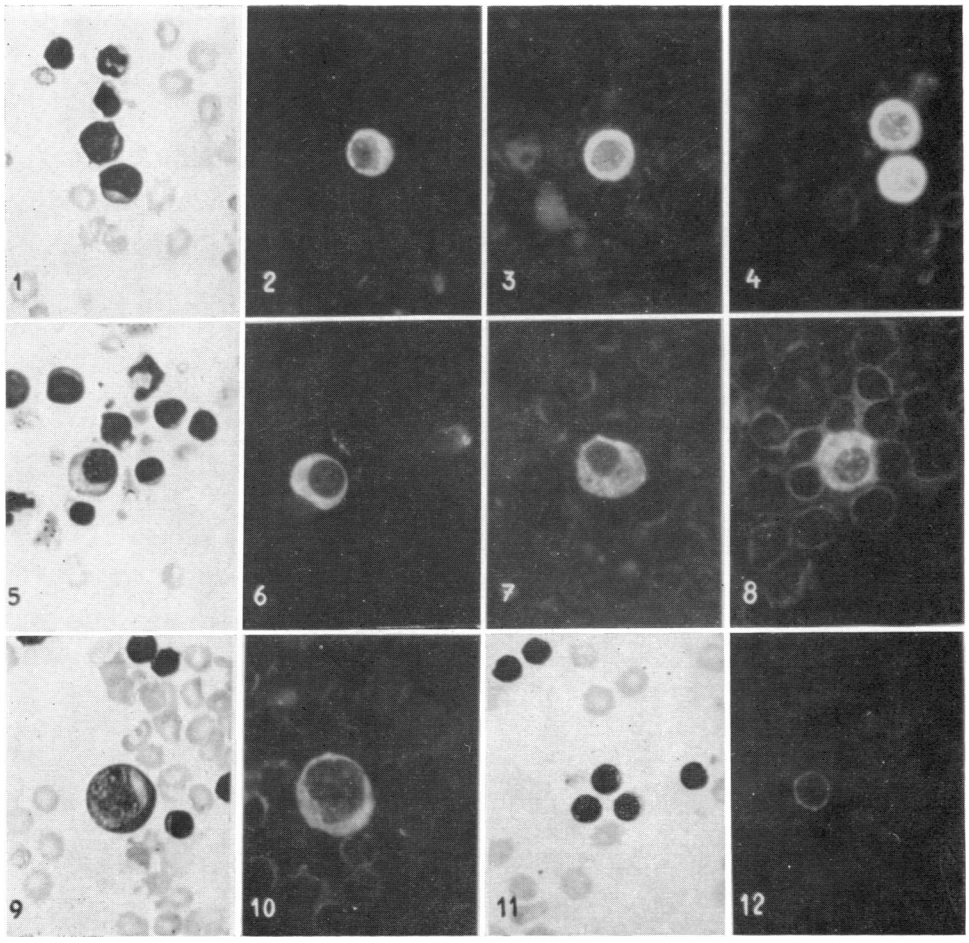

FIG. 3. Spleen. *Medium-sized lymphocytes:* (1) Giemsa stained. Fluorescence positive for: (2) IgG; (3) IgA; (4) IgM. *Plasma cells:* (5) Giemsa stained. Fluorescence positive for: (6) IgG; (7) IgA; (8) IgM. *Large lymphocytes:* (9) Giemsa stained; (10) IgM-positive fluorescence. *Small lymphocytes:* (11) Giemsa stained; (12) IgM-positive fluorescence. × 500.

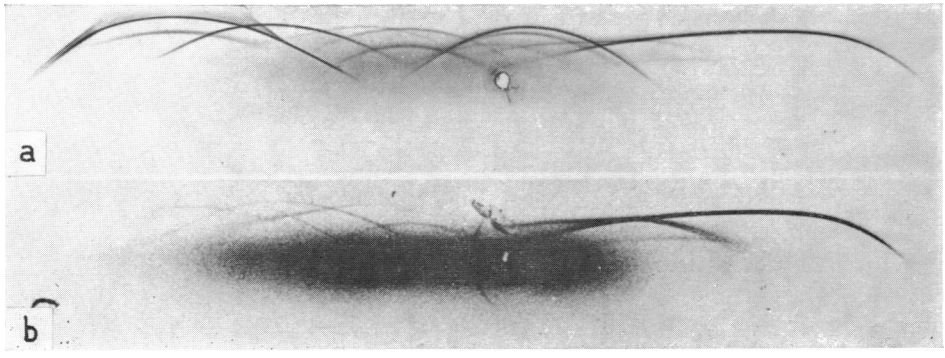

FIG. 4. Normal bone marrow. (a) Immunoelectrophoretic pattern of carrier serum and culture fluid developed with horse anti-human antiserum; (b) autoradiograph with labelling of IgG, IgA and IgM lines. Note spot of precipitated labelled haemoglobin.

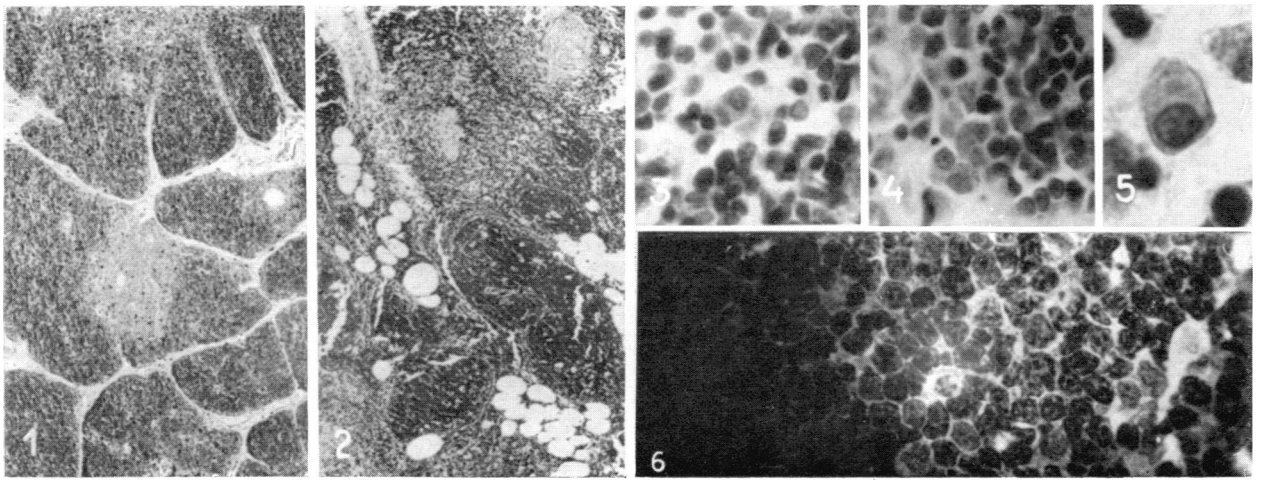

FIG. 5. Normal thymus. H & E stained biopsy sample from :(1) 4-year-old infant, normal structure; (2) 42-year-old female, cortex and medulla partly replaced by fat (× 40). Methyl green pyronin stained; (3) plasma cells in medulla (× 450); (4) plasma cells in interstitial connective tissue (× 450); (5) plasma cell (× 1150); Immunofluorescent stained: (6) IgG positive plasma cell (× 500).

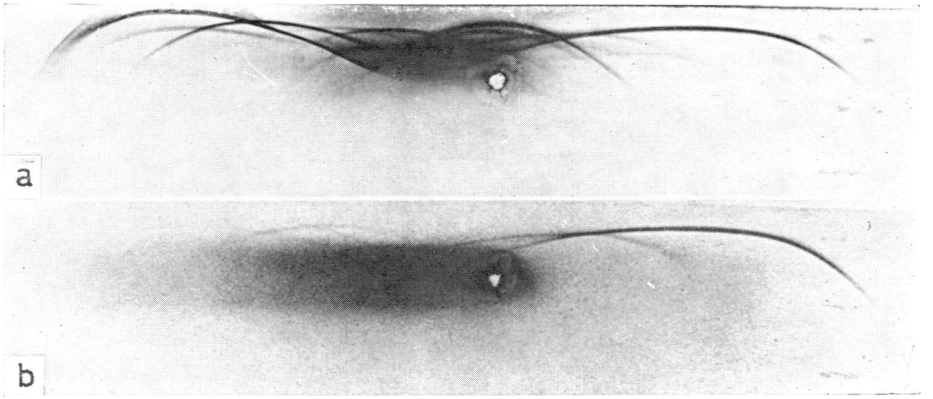

FIG. 6. Normal thymus. (a) Immunoelectrophoretic pattern of carrier serum and culture fluid developed with horse anti-human antiserum; (b) autoradiograph with labelling of IgG and IgA lines.

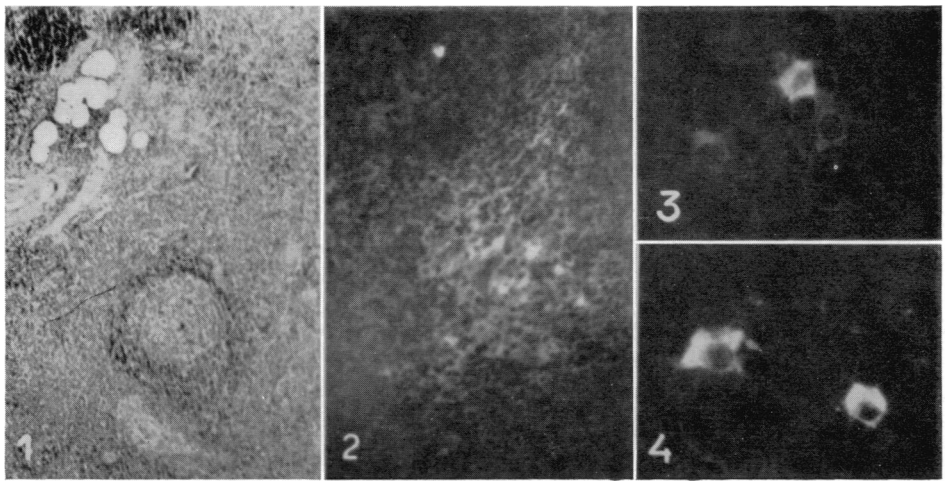

FIG. 7. Thymus—systemic lupus erythematosus. (1) Germinal centre in medulla, H & E stained (×40); (2) IgM-positive fluorescence of lymphoid cells in germinal centre (×100); (3) IgG-positive plasma cell (×500); (4) IgM-positive plasma cells (×500).

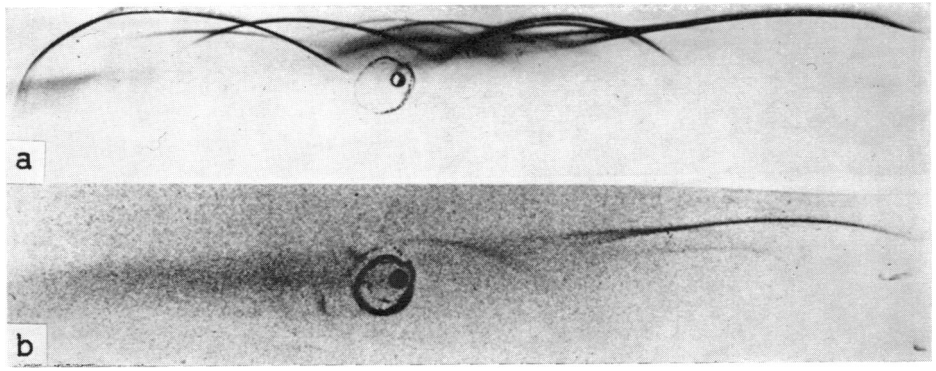

FIG. 8. Thymus—systemic lupus erythematosus. (a) Immunoelectrophoretic pattern of carrier serum and culture fluid developed with horse anti-human antiserum; (b) autoradiograph with labelling of IgG, IgA and IgM lines.

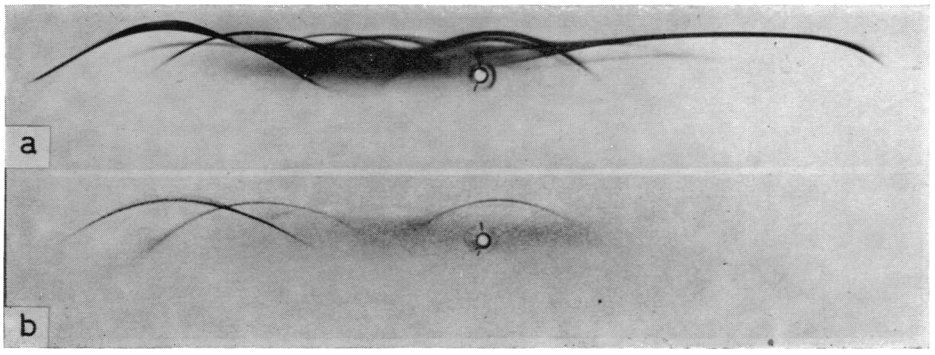

FIG. 9. Liver. (a) Immunoelectrophoretic pattern of carrier serum and culture fluid developed with horse anti-human antiserum; (b) autoradiograph with labelling of albumin, α_{1A}-globulin, siderophilin line and very weak IgG line.

the suspensions an occasional IgM-positive lymphocyte was seen, but no IgM-positive plasma cells.

In *autoimmune disease* the sample obtained from the patient with lupus erythematosus showed a cortex partly replaced by fat tissue and a medulla with a few germinal centres (Fig. 7). In the sample of the patient with myasthenia gravis, most of the right lobe was occupied by a thymoma, which enclosed remnants of thymus tissue containing germinal centres; the other lobe, which was also removed, had a normal histological appearance.

The *autoradiographs* of both pathological thymus samples demonstrated that IgG, IgA and also IgM were synthesized (Fig. 8). The culture of the non-pathological lobe of the thymus of the patient with a thymoma synthesized only IgG and IgA.

Immunofluorescent staining for IgM in the frozen sections of both pathological samples showed several strongly positive plasma cells in the medulla; the samples from the lupus erythematosus patient contained germinal centres with IgM-positive cells (Fig. 7). The sample of thymoma tissue used for frozen sections contained no germinal centres. The sections of the normal lobe of this patient showed only two IgM-positive plasma cells. The staining for IgG showed an occasional positive plasma cell.

OTHER ORGANS

The liver samples showed the synthesis of a trace of IgG, no IgA or IgM and the labelling of other proteins such as albumin, α_{1A}-globulin and siderophilin (Fig. 9). Three of the four lung samples yielded a very small amount of IgG and IgA, but no IgM. One of the three thyroid samples synthesized only a small amount of IgG, the other immunoglobulins were absent.

DISCUSSION

The incubation of normal lymph nodes, spleen and bone marrow has shown that these tissues can synthesize IgG, IgA and IgM. The cells which may be regarded as involved in this synthesis, because of their cytoplasmatic fluorescence, are medium-sized and large lymphocytes, and plasma cells. These cells were found to be positive for IgG, IgA or IgM. The small lymphocytes behaved exceptionally because they were positive only for IgM. This point will be discussed in detail in a subsequent paper (van Furth *et al.*, 1966b).

It was remarkable that in normal bone marrow samples only plasma cells contained immunoglobulins, although bone marrow contains scattered lymphoid cells. Therefore in bone marrow only plasma cells are responsible for the immunoglobulin synthesis. This conclusion is supported by the almost complete absence of immunoglobulin formation by the bone marrow samples in agammaglobulinaemia.

Although the absence of fluorescent lymphocytes in the bone marrow samples may be due to the relatively small number of these cells as compared to the number present in such lymphopoietic organs as the lymph nodes and spleen, another explanation can also be considered. Several investigators have reported that bone marrow, in contrast to the spleen and lymph nodes, does not form antibodies during a primary response (Gengozian, Makinodam and Shekarchi, 1961; Langevoort, Asofsky, Jacobson, de Vries and Thorbecke, 1963; Friedman, 1964), but participates only in the secondary response (Thorbecke and Keuning, 1953, 1956; Thorbecke, Asofsky, Hochwald and Siskind, 1962). To explain these findings it has been supposed that the antibody-producing cells do not originate in the bone marrow but are derived from other reticulo-endothelial organs (Langevoort *et al.*, 1963). Our results obtained with immunofluorescent staining may be consistent with

this view. In the spleen and the lymph nodes primary antibody formation is associated with the proliferation of immature plasma cells, and suggestive evidence has been adduced that these cells descend from small lymphocytes (Langevoort, Keuning, van der Meer, Nieuwenhuis and Oudendijk, 1961; Langevoort, 1961, 1963; van Buchem, 1962). In both organs the cells that contain immunoglobulins are medium sized and large lymphocytes and cells of the plasma-cell series. These cells are presumably in the process of antibody formation. The complete absence of fluorescent lymphocytes in the bone marrow may indicate that the lymphoid elements in this organ do not participate in a primary antibody response. The fluorescent plasma cells, which synthesize immunoglobulins, then would not arise from the lymphoid elements of the bone marrow, but would conceivably be immunoglobulin-synthesizing cells that are derived from other lymphopoietic organs, and reach the bone marrow via the circulation.

Cultures of normal thymus tissue obtained from infants and adults differed from the pattern generally found in lymphoid tissue by the complete absence of IgM. The thymus synthesized only IgG in a distinct amount and a very small amount of IgA. These findings agreed with the presence of IgG- and IgA-positive plasma cells and medium-sized lymphocytes and the absence of plasma cells positive for IgM. The plasma cells were mainly localized in the interlobar connective tissue and the medulla, in close relation to the penetrating vessels. These observations agree with the results of thymus cultures from various species of animals, which also show the production of a very small amount of immunoglobulin (Askonas and White, 1956; Thorbecke, 1960; Asofsky and Thorbecke, 1961; Thorbecke and Cohen, 1964) and with the immunofluorescent studies of White and Marshall (1962).

In contrast to other lymphoid organs, there is little or no antibody formation and no plasma cell proliferation or formation of germinal centres in the thymus after systemic administration of antigens (Fagraeus, 1948; Thorbecke and Keuning, 1953, 1956; Askonas and White, 1956). Direct injection of antigens into this organ, however, induces the formation of both germinal centres and plasma cells as well as the production of antibodies (Marshall and White, 1961). Also in transplantation experiments the lymphoid cells of the thymus have been shown to be immunologically competent (Stoner and Hale, 1955; Dixon, Weigle and Roberts, 1957; Vos, de Vries, Collenteur and van Bekkum, 1959; Stoner and Bond, 1963). To explain the absence of antibody formation it has been postulated that the normal thymus possesses a barrier against the entry of antigens from the blood stream (Marshall and White, 1961) and evidence for this has been adduced from electron-microscope studies (Clark, 1963; Weiss, 1963).

With respect to our results it is necessary to consider the origin of the cells in the thymus, which are responsible for the synthesis of immunoglobulins. Two possibilities will be discussed: plasma cells may either originate in the thymus or these cells may derive from other lymphoid organs and be trapped by the thymus from the circulation. Assuming that all immunoglobulins are associated with antibody activity, the first supposition entails that the thymus participates in antibody formation, but only for the IgG and IgA types. This would mean an exceptional situation because, as in the other lymphoid organs, the synthesis of IgM would also be expected. The first supposition would therefore imply that the thymus contains cells exclusively capable of producing IgG and IgA. Supporting evidence cannot be adduced for this possibility and an analogous situation is not known. The second consideration, which concerns the trapping of cells, is supported by the localization of these cells in the more vascularized areas. These cells would accordingly derive from

the peripheral blood which contains immunoglobulin synthesizing cells (van Furth, 1964; van Furth *et al.*, 1966b). Since approximately equivalent amounts of radioactive immunoglobulin result in identical autoradiographic images (van Furth, 1964, 1966), comparison of the autoradiographs of peripheral blood and thymus samples should show similar pictures. In fact they show the same intensity of the IgG line but differences in the pictures of the IgM line (van Furth, 1964); peripheral blood reveals a distinct IgM line, which is consistently absent in thymus cultures. These results agree with the almost complete absence of IgM-synthesizing cells in the thymus, in contrast to the peripheral blood (van Furth, 1964; van Furth *et al.*, 1966b). Therefore, if the thymus traps immunoglobulin-synthesizing cells from the peripheral blood, there may either be a selection for IgG synthesizing cells or a difference in the lifespan of IgG- and IgM-synthesizing cells. As it has been suggested that the IgM-synthesizing cells may be short-lived cells with a limited capacity to produce immunoglobulins as compared to the IgG-synthesizing cells (Bauer, Mathies and Stavitsky, 1963), it seems likely that the thymus lodges only the older, long-lived, IgG-synthesizing cells.

The patterns of immunoglobulins synthesized by lymphoid elements in parenchymatous organs such as the lung, thyroid gland and liver, show like the thymus no synthesis of IgM, but in contrast only a very limited, if any, synthesis of IgG and IgA. Although the same considerations as hold for the thymus might be applied here, comparison is not justified because the synthesis of IgG, which normally far exceeds that of IgM, was barely detectable in these samples.

Thymus biopsy samples obtained from patients with an autoimmune disease revealed a different pattern as they produced IgM as well as IgG and IgA. The immunofluorescent staining also showed, in contrast to the normal thymus, IgM-positive plasma cells and germinal centres. It is likely that the IgM synthesis by the thymus in autoimmune diseases is associated with the pathological changes in the thymus. This is supported by the absence of IgM synthesis in the sample of the normal thymus lobe from the patient with myasthenia gravis. Plasma cells and germinal centres in the medulla have been observed in autoimmune diseases in mice of the NZB strain (Bielschowsky, Helyer and Howie, 1959; Holmes, Gorrie and Burnet, 1961; Burnet and Holmes, 1962) and in man in myasthenia gravis (White and Marshall, 1962), rheumatoid arthritis (Burnet and Mackay, 1962) and systemic lupus erythematosus (Mackay and de Gail, 1963; Hutchins and Harvey, 1964). Our finding that the immunoglobulin synthesis by the pathological thymus is similar to that in other lymphoid organs, which participate in immune response, raises the question whether in autoimmune diseases the thymus synthesizes autoantibodies. A model in which this question could be studied is provided by the Bielschowsky strain of mice.

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