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The Formation of Immunoglobulins by Human Tissues *in vitro*

IV. CIRCULATING LYMPHOCYTES IN NORMAL AND PATHOLOGICAL CONDITIONS

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Summary. The formation of immunoglobulins by circulating lymphocytes was studied by three techniques: (1) Autoradiographic analysis of the immunoglobulins synthesized during the incubation of cell suspensions in a medium with radioactive amino acids; (2) direct immunofluorescent staining; and (3) examination of the cellular morphology.

Lymphocytes of the normal peripheral blood were found to synthesize a distinct amount of IgG and smaller amounts of IgA and IgM. Cells of the thoracic-duct lymph synthesized distinct amounts of all three immunoglobulins. A similar pattern was found in infectious mononucleosis and rubella. In infectious mononucleosis the significantly increased synthesis of IgM during the first 10 days of illness led to the supposition that this result may be due to primary antigenic stimulation. The pattern in chronic lymphatic leukaemia is characterized by the consistent absence of IgA and the labelling of IgG, mainly the medium to high mobility part, and of IgM. In agammaglobulinaemia a trace of IgG and IgA was found in one case; the other was entirely negative.

The immunofluorescent staining showed that in all samples some of the medium-sized lymphocytes contain IgG, IgA or IgM. Peripheral blood samples taken during an infectious mononucleosis or rubella infection and thoracic duct lymph revealed also positive large lymphocytes and plasma cells.

A remarkable observation was the weak fluorescence of small lymphocytes which were exclusively positive for IgM. It is postulated that these small lymphocytes indicate their initial synthesis of IgM antibodies when engaged in primary response.

INTRODUCTION

The immunological activities of circulating lymphocytes can be distinguished in homotransplantation reactions, delayed hypersensitivity and antibody formation. Convincing evidence has been presented concerning the first two of these functions, but knowledge concerning the formation of antibodies by circulating lymphocytes is still limited (see review by Gowans and McGregor, 1965). It has been shown that the lymphoid cells of the thoracic-duct lymph in the rabbit can synthesize antibodies *in vitro* (Wesslén, 1952; Hollander and Danielsson, 1962), but the capacities of the lymphocytes of the peripheral blood were largely unknown. Recently it has been shown in animals that lymphocytes of

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the peripheral blood are able to synthesize immunoglobulins (Thorbecke, Asofsky, Hochwald and Jacobson, 1964) and antibodies (Hulliger and Sorkin, 1963; Friedman 1964) *in vitro*.

Transformation of small lymphocytes into large pyroninophilic cells capable of division has been demonstrated in diffusion chamber cultures of stimulated thoracic-duct lymphocytes of the rabbit (Holub, 1960, 1962) and in culture studies of human peripheral blood lymphocytes in the presence of phytohaemagglutinin (Mackinney, Stohlman and Brecher, 1962; Elves and Wilkinson, 1962; Elves and Israëls, 1963), specific antigens (Schreck, 1963; Elves, Roath and Israëls, 1963; Cowling, Quaglino and Davidson, 1963; Pearmain, Lycette and Fitzgerald, 1963; Hirschhorn, Back, Kolodny, Firschen and Haslem, 1963; Ling and Husban, 1964; Holland and Mauer, 1964), homologous leucocytes (Bain, Vas and Lowestein, 1963, 1964) or leucocyte antibodies (Gräsbeck, Nordman and de la Chapelle, 1963, 1964). The production of immunoglobulins and the presence of antibodies in these transformed cells (Bach and Hirschhorn, 1963; Elves, Roath, Taylor and Israëls, 1963; Bartfield and Juliar, 1964) has also been reported, but with insufficient evidence about the specificity of the observations.

The present study was undertaken to investigate the capability for immunoglobulin synthesis of circulating lymphocytes in man and to determine the morphology of the cells that may be engaged.

To this end, thoracic-duct lymph and peripheral blood from healthy donors and patients suffering from such diseases as infectious mononucleosis, rubella, chronic lymphatic leukaemia and agammaglobulinaemia were investigated. The methods applied were a technique for demonstrating the synthesis of serum proteins *in vitro* (Hochwald, Thorbecke and Asofsky, 1961), direct immunofluorescent staining, and the morphological study of the cells.

MATERIALS AND METHODS

A detailed description of the preparation of the lymphocyte cultures and the immunoelectrophoretic and autoradiographic analysis of the synthesized immunoglobulins, the immunofluorescent staining, and the morphological examination has been given in a previous paper (van Furth, Schuit and Hijmans, 1966a), The immunofluorescent staining was performed only on lymphocytes prior to incubation. The lymphocytes were classified, according to the criteria of Nossal and Mäkelä (1961): small lymphocytes (diameter $\leqslant 7\,\mu$), medium-sized lymphocytes (diameter 8–9 μ), and large lymphocytes (diameter $\geqslant 10\,\mu$).

Lymph was collected from five patients who had undergone thoracic duct drainage for various reasons, such as hepatic cirrhosis (two), pericarditis (one), intestinal carcinoma (one), lung abscess (one). Lymph was also obtained from a normal individual, from a patient with an accidental fistula after thyroidectomy, and from a case of chylothorax after cardiac surgery. The lymphocytes of the thoracic-duct lymph were concentrated by centrifugation and cultured as described previously.

Lymphocytes from peripheral blood were obtained from nine healthy donors. Because the samples contained a large number of granulocytes, the latter were removed with a nylon fibre filter according to Greenwalt, Gajenoski and McKenna (1963). In addition to the routine procedures, two cultures were prepared with the standard medium but without the addition of ovalbumin. These two cultures served as controls to determine

whether a possible antigenic stimulation by ovalbumin could be excluded. All cultures were prepared without the addition of phytohaemagglutinin or specific antigens. Immunofluorescent staining was performed on the majority of these cultured samples, but numerous additional samples from healthy donors were also studied.

Blood samples were obtained from eighteen adults suffering from infectious mononucleosis. Because of the relatively large amount of lymphoid cells in these blood samples, the granulocytes were not removed. Immunofluorescent staining was done in all these samples, and also in samples from twelve other patients in which no culture experiments were performed. The criteria for the diagnosis were the clinical features (Hoagland, 1960a, b; Wintrobe, 1961), the morphological blood picture, and a heterophile antibody titre of $\geqslant 1:32$ after absorption with guinea-pig kidney. Abnormal liver-function tests, regarded as a part of the syndrome, gave support to the diagnosis (Dunnet, 1963). The duration of the illness was counted from the onset of symptoms such as malaise, sore throat, or fever. The incubation time, which is generally reported to be 5–15 days or more (Hoagland, 1955), was not taken into account.

Blood samples from two adult patients with classical rubella were also investigated.

The immunoglobulin formation in chronic lymphatic leukaemia was studied in blood samples obtained from nine adult patients, who were receiving no specific therapy, e.g. radiotherapy, chemotherapy or corticosteroids, at the time of investigation, and from one patient who was being treated with a low dose of corticosteroids. Because of the relatively small number of granulocytes in the peripheral blood, these cells were not removed.

The lymphocytes of the peripheral blood from two patients with idiopathic agammaglobulinaemia were investigated; one was a female (B) aged 25 with congenital, sporadic, non-sex-linked form of agammaglobulinaemia and the other a male (K) of 42 years, suffering from the acquired form of primary hypogammaglobulinaemia. According to the double-diffusion test in agar gel, only trace amounts of all three immunoglobulins were present in both sera.

RESULTS

THORACIC-DUCT LYMPH

Morphologically, the lymphoid cells of the two samples collected in our laboratory were found to contain about 5 per cent small lymphocytes, 84 per cent medium-sized lymphocytes, 4 per cent plasma cells, and 3·5 per cent lymphoid blast cells.

The *cultures* of all eight cell suspensions had synthesized a distinct amount of all three immunoglobulins (Fig. 1). The average score of the gradation of the autoradiographic line was ++ for IgG, as well as for IgA and IgM.

Immunofluorescent staining showed a considerable number of medium-sized cells, many large lymphocytes, with a rim of brilliantly-fluorescent cytoplasm, and brightly-fluorescent cells belonging to the plasma-cell series, all positive for IgG, IgA or IgM. Furthermore, a large number of weakly-fluorescent small lymphocytes positive only for IgM were observed (Fig. 2).

NORMAL PERIPHERAL BLOOD

Differential counts of the *cytological* preparations of the samples obtained from healthy donors gave a mean of 28 per cent small lymphocytes (range 21–32 per cent), 71 per cent medium-sized lymphocytes (range 67–79 per cent), and 0–1 per cent cells of the plasma-cell series. After filtration of the lymphocyte suspension the number of small lymphocytes

in the samples decreased slightly with respect to the medium-sized cells; this shift was probably caused by trapping of the former in the nylon-fibre column, since erythrocytes, always present in small numbers, showed a similar reduction after filtration.

The *culture* fluids demonstrated a distinct synthesis of IgG and small amounts of IgA and IgM (Fig. 3, Table 1). The minimum number of cells required to synthesize a detectable amount of immunoglobulins is of the order of $0{\cdot}1 \times 10^8$, but optimal results were obtained when more than three times this number was present. The samples cultured in a medium without ovalbumin also showed synthesis of immunoglobulins in comparable amounts.

TABLE 1

RESULTS OF AUTORADIOGRAPHY OF NORMAL PERIPHERAL BLOOD LYMPHOCYTE CULTURES

Culture No.	Mononuclear cells ($\times 10^8$)	Autoradiography*		
		IgG	IgA	IgM
370	0·07	−	−	−
278	0·08	(+)	(+)	−
275	0·12	+	+	+
371†	0·14	+	−	−
433	0·30	(+)	(+)	−
277	0·36	++	+	+
276	0·76	++	+	+
271	0·80	++	+	+
476§	1·10	+	+	+
434‡	1·20	+	+	(+)
477§	1·80	++	+	+

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

† From same sample as No. 370.

‡ From same sample as No. 433.

§ Samples incubated in a medium without ovalbumin.

Immunofluorescent staining of peripheral blood lymphocytes for IgG and IgA showed a few medium-sized lymphocytes with clearly fluorescent cytoplasm. The staining with anti-IgM conjugate showed a small number of medium-sized lymphocytes with clearly fluorescent cytoplasm and a varying number of weakly-fluorescent small lymphocytes (Fig. 4).

INFECTIOUS MONONUCLEOSIS

Morphologically, the eighteen blood samples used for the incubation experiments showed a mean of 7·7 per cent small lymphocytes (range 3–14 per cent), 56·3 per cent medium-sized lymphocytes (range 30–72 per cent), 0·8 per cent large lymphocytes (range 0–2 per cent), 34·8 per cent atypical cells (range 16–59 per cent) and 0·4 per cent plasma cells (range 0–1 per cent).

The *autoradiographs* of the cultured samples generally showed labelling of all three immunoglobulins (Fig. 5). Because the intensity of the autoradiography could not be related to the number of cultured cells or the number of atypical cells the samples were arranged according to the day of illness at the time of sampling. The results for the first 10 days show a marked synthesis of IgG, IgA, and IgM. The samples taken from the eleventh to the fourteenth days show a diminished production of immunoglobulins, and the samples

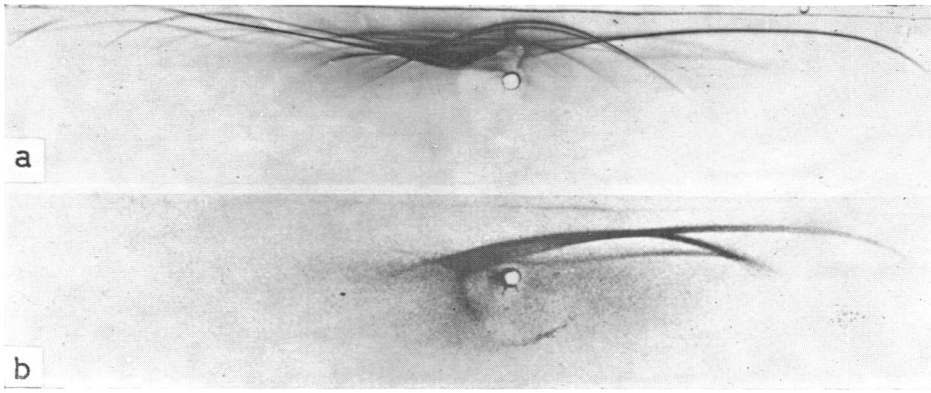

FIG. 1. Thoracic-duct lymph. (a) Immunoelectrophoretic pattern of carrier serum and culture fluid developed with horse anti-human antiserum; (b) autoradiograph with labelling of IgG, IgA and IgM lines.

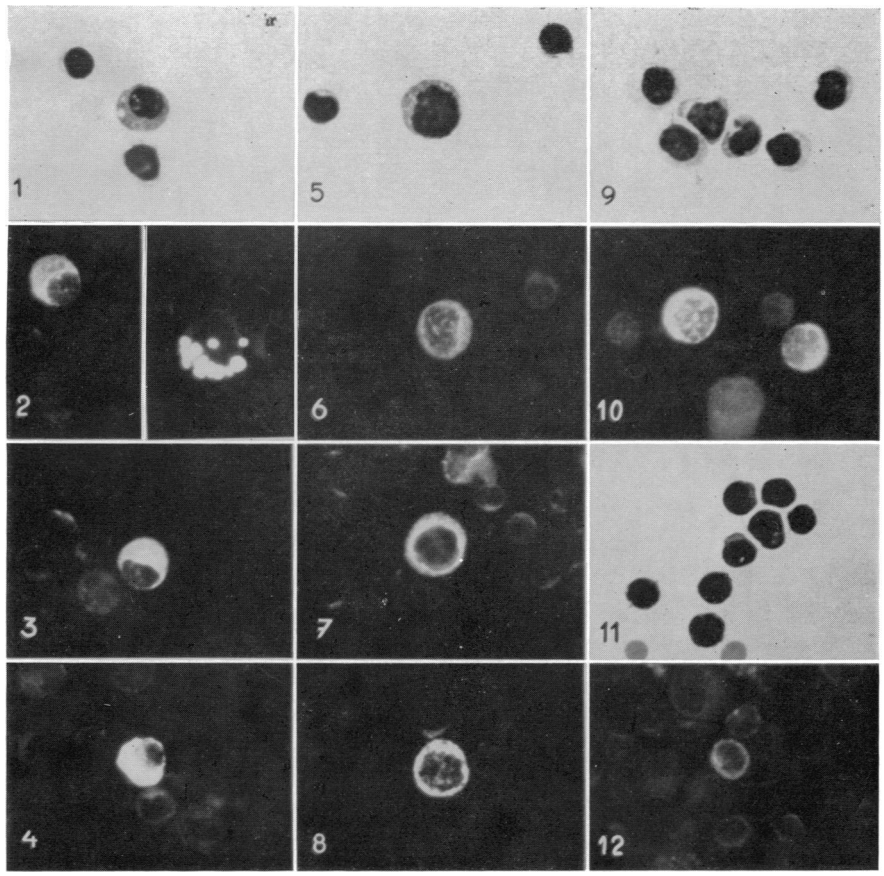

FIG. 2. Thoracic-duct lymphocytes. *Plasma cells*: (1) Giemsa stained. Fluorescence positive for: (2) IgG, note Russell body; (3) IgA; (4) IgM. *Large lymphocytes*: (5) Giemsa stained. Fluorescence positive for: (6) IgG; (7) IgA; (8) IgM. *Medium-sized lymphocytes*: (9) Giemsa stained; (10) IgM-positive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

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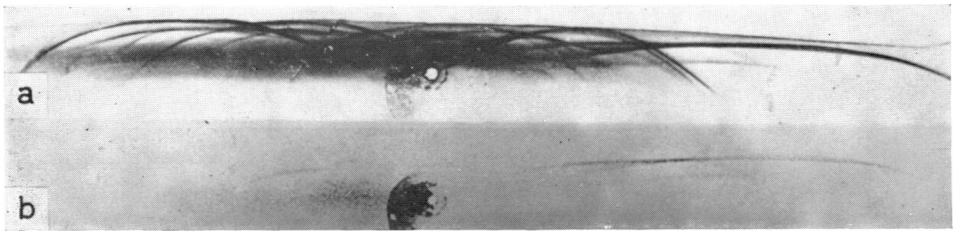

FIG. 3. Normal peripheral blood. (a) Immunoelectrophoretic pattern of carrier serum and culture fluid (No. 276) developed with horse anti-human antiserum; (b) autoradiograph with labelling of IgG, IgA and IgM lines.

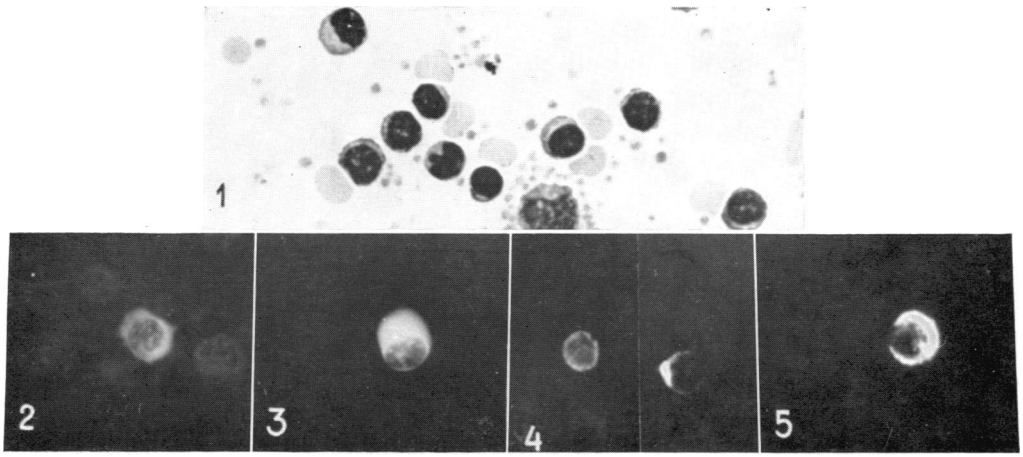

FIG. 4. Normal peripheral blood lymphocytes. (1) Giemsa stained medium-sized and small lymphocytes. *IgG-positive fluorescence* in: (2) medium-sized lymphocyte; (3) plasma cell. *IgM-positive fluorescence* in: (4) small lymphocytes; (5) medium-sized lymphocyte. × 500.

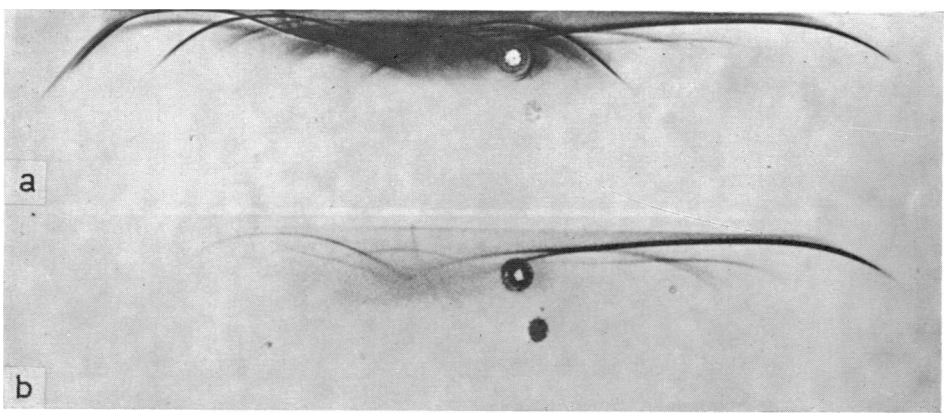

FIG. 5. Infectious mononucleosis. (a) Immunoelectrophoretic pattern of carrier serum and culture fluid (No. 75) developed with horse anti-human antiserum; (b) autoradiograph with labelling of IgG, IgA and IgM lines.

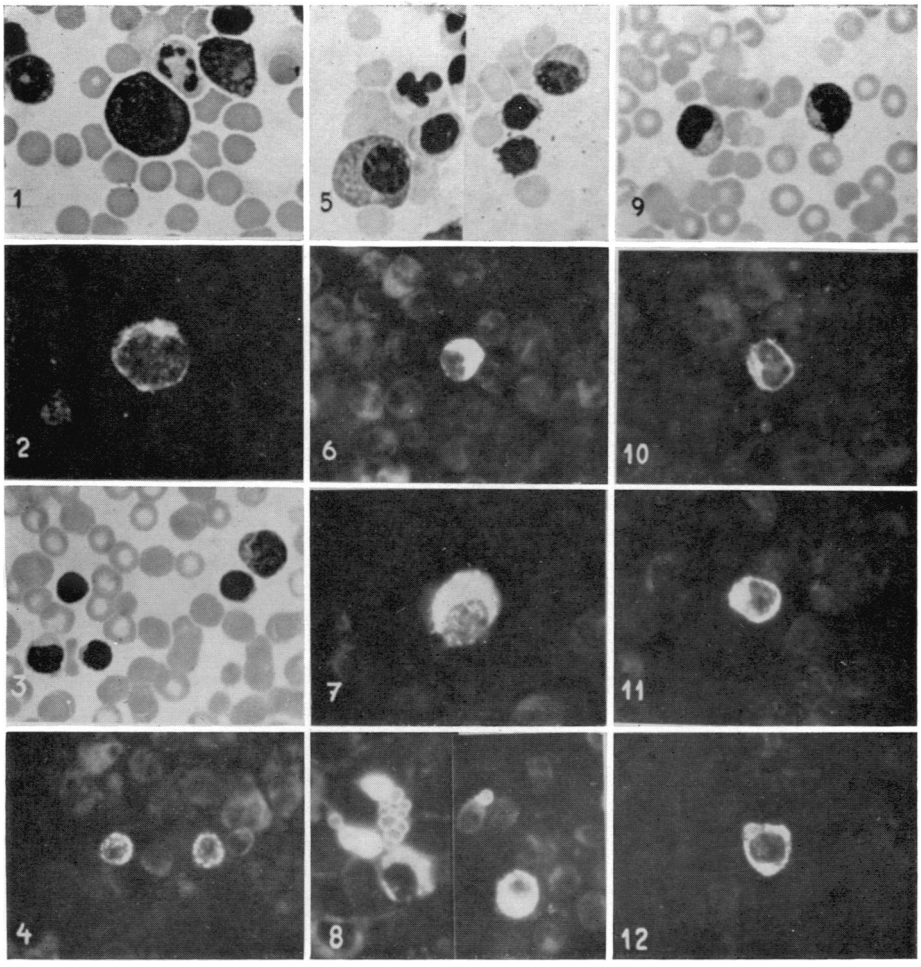

FIG. 6. Peripheral blood lymphocytes—infectious mononucleosis. *Atypical cells*: (1) Giemsa stained; (2) IgM-positive fluorescence. *Small lymphocytes*: (3) Giemsa stained; (4) IgM-positive fluorescence. *Plasma cells*: (5) Giemsa stained. Fluorescence positive for: (6) IgG; (7) IgA (8) IgM, note Russell body. *Medium-sized lymphocytes*: (9) Giemsa stained. Fluorescence positive for: (10) IgG; (11) IgA; (12) IgM. × 500.

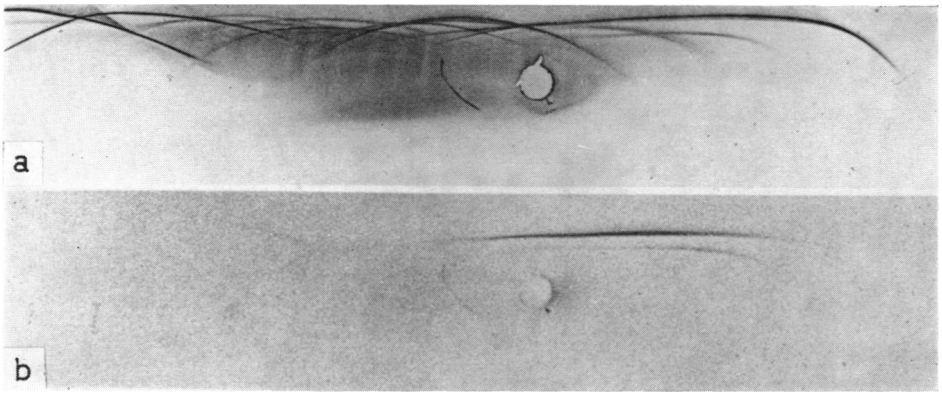

FIG. 7. Peripheral blood lymphocytes—chronic lymphatic leukaemia. (a) Immunoelectrophoretic pattern of carrier serum and culture fluid (No. 157) developed with horse anti-human antiserum; (b) autoradiograph with labelling of the IgG and IgM lines. Note the preferential labelling of the medium to high mobility part of the IgG line.

TABLE 2

RESULTS OF AUTORADIOGRAPHY OF PERIPHERAL BLOOD LYMPHOCYTE CULTURES FROM PATIENTS WITH INFECTIOUS MONONUCLEOSIS

Culture No.	Day of illness	Heterophile antibody titre	Liver function test†	Cultured mononuclear cells ($\times 10^8$)	Autoradiography*		
					IgG	IgA	IgM
365	4	1/224	+	0·2	(+)	(+)	(+)
338	5	1/56	+	0·6	+	+	+
331	5	1/112	−	0·5	++	++	++
99	7	1/224	+	1·3	++	++	++
75	8	1/112	+	1·3	+++	++	++
258	8	1/56	+	0·5	++	++	++
346	9	1/32	+	0·6	+	−	+
237	10	1/128	+	0·5	++	++	++
319	11	1/224	+	1·4	(+)	(+)	−
252	12	1/224	+	0·3	(+)	−	−
233	13	1/512	+	1·1	(+)	−	(+)
266	13	1/1024	+	0·8	+	(+)	−
214	14	1/224	−	1·2	−	−	−
63	14	1/224	±	1·7	−	−	−
268	16	1/512	+	0·5	++	+	+
333	16	1/112	+	0·5	+	+	+
251	17	1/512	+	1·4	+	+	−

* For symbols see Table 1.
† +, Abnormal; −, normal.

taken after the fourteenth day of illness show an autoradiographic pattern identical to that of normal peripheral blood (Table 2). There was only one definite exception; one sample taken on the sixth day was entirely negative although a maximum yield was expected. This result was not included in the Table because the samples taken on the thirteenth and twenty-first day from this patient were also negative. The reason for this exceptional behaviour is not clear but may depend upon the properties of the lymphocytes of this patient. The immunoglobulins in the serum appeared to be normal.

The *immunofluorescent* staining revealed several types of positive cells. The IgG, IgA and IgM positive cells were medium-sized and large lymphocytes, and a varying number of brilliantly-fluorescent plasma cells; occasionally an IgM-positive Russell body was found. A small number of medium-sized and large cells with a clearly-fluorescent cytoplasm and an irregularly-shaped nucleus, most probably the atypical cells, were found positive for IgM or IgG. Furthermore, numerous small lymphocytes with a rim of weakly-fluorescent cytoplasm, which were exclusively positive for IgM, were seen (Fig. 6). During about the first 10 days of illness the samples were positive for all three categories of immunoglobulins, but thereafter the staining for IgG and IgA showed only occasionally a positive cell or was negative and the number of IgM-positive cells showed a marked reduction.

RUBELLA

Both of the samples contained small lymphocytes (5 and 9 per cent), medium-sized lymphocytes (35 and 50 per cent), large lymphocytes (4 and 1 per cent), atypical lymphocytes (53 and 38 per cent), and plasma cells (3 and 2 per cent).

The *autoradiographic* image of both cultures showed an intense labelling of the IgG, IgA and IgM lines.

The *immunofluorescent staining*, performed only for IgM, showed numerous medium-sized and large lymphocytes and plasma cells with a brilliant fluorescence and small lymphocytes with a weakly-fluorescent cytoplasm.

CHRONIC LYMPHATIC LEUKAEMIA

The differential count of the samples gave a mean of 8·7 per cent small lymphocytes (range 3–17·5 per cent), 72·6 per cent medium-sized lymphocytes (range 26–89·5 per cent), 2·2 per cent large lymphocytes (range 0–5 per cent) and 16·5 per cent blast cells (range 5·5–14·5 per cent with one extreme value of 65 per cent).

Because chronic lymphatic leukaemia is often accompanied by changes in the immunoglobulins, the γ-globulin content was determined and immunoelectrophoretic analysis of the serum was carried out. The results show that of the ten patients, six had a hypogammaglobulinaemia, three were in the normal range and one patient had an increased γ-globulin content. The immunoelectrophoretic analysis revealed that in the majority of the cases one or more of the immunoglobulins were distinctly decreased. However, in one case (248) a cryomacroglobulinaemia was present and another (323) showed a high-mobility IgG paraproteinaemia. In two other cases, the high-mobility part of the IgG precipitation line was slightly increased, as judged from the immunoelectrophoretic pattern prepared with an anti-IgG antiserum.

TABLE 3

RESULTS OF AUTORADIOGRAPHY OF LYMPHOCYTE CULTURES FROM PATIENTS WITH CHRONIC LYMPHATIC LEUKAEMIA

Culture No.	Serum γ-globulin (g/100 ml)	Cultured mononuclear cells ($\times 10^8$)	Autoradiography*		
			IgG	IgA	IgM
316	0·42	1·0	++	−	+
248	0·22	1·8	++	−	++
308	1·67	2·6	+†	−	+
339	0·64	2·9	+†	−	(+)
431	0·54	3·2	+†	−	+
318	0·33	3·4	+	+†	−
297	0·90	3·5	+†	−	+
323	0·71	4·4	(+)	−	−
157	0·18	5·2	++†	−	+
238	0·80	5·5	+	−	+

* For symbols see Table 1.
† Medium to high mobility part mainly labelled.

The *autoradiographic* pattern in chronic lymphatic leukaemia showed a characteristic picture: labelling of the IgG line in all the samples and in eight out of ten samples a labelled IgM line; however, IgA was absent in all samples except for one, in which only the high-mobility part of the IgA line was labelled (Table 3). It is noteworthy that in six samples the labelling was mainly localized in the medium- to high-mobility part of the IgG precipitation line, while labelling of the low-mobility part was either very weak or entirely absent (Fig. 7).

The *immunofluorescent staining* for IgG showed a few weakly or clearly-fluorescent medium-sized cells. Fluorescence for IgA was absent except in one sample (No. 157) in which a few weakly-positive cells were observed. A varying number of IgM-positive cells was found in all samples; these appeared to be a small number of medium-sized cells with a distinct rim of clearly-fluorescent cytoplasm which could not be easily distinguished in these

preparations as either lymphocytes or blast cells, and a large number of small lymphocytes with a small rim of weakly-fluorescent cytoplasm.

AGAMMAGLOBULINAEMIA

The lymphocyte counts of the peripheral blood of both patients were in the normal range.

The lymphocyte *culture* from patient B was consistently negative for all three immunoglobulins; the culture of patient K showed traces of both IgG and IgA.

The *immunofluorescent staining* of both samples gave entirely negative results for IgG and IgA and showed only a very small number of small lymphocytes weakly-positive for IgM.

DISCUSSION

These incubation studies with circulating lymphocytes of peripheral blood and thoracic duct lymph clearly show that these cells are capable of producing immunoglobulins. Samples of peripheral blood lymphocytes from healthy donors, which contain predominantly medium-sized lymphocytes and hardly any plasma cells, show a distinct synthesis of IgG and smaller amounts of IgA and IgM. These cells are mainly derived from two sources, the thoracic duct lymph and the white pulp of the spleen. The immunofluorescent staining showed that some of these cells are immunoglobulin-producing lymphocytes, which have conceivably been recently stimulated by antigen in the lymphoid organs, but that there is also a considerable number of lymphocytes not engaged in the synthesis of immunoglobulins at the time of examination.

The present results were obtained in the absence of phytohaemagglutinin or specific antigens; the results of the control experiments make it very unlikely that ovalbumin acted as antigenic stimulus. With respect to reports mentioned in the introduction, further studies are required to investigate whether as well as transforming peripheral blood lymphoctyes, phytohaemagglutinin or antigen induces also an increased synthesis of immunoglobulins or the formation of specific antibodies *in vitro*.

Comparison of the autoradiographic images from the thoracic-duct lymph samples, which showed a very distinct labelling of three immunoglobulins, with the patterns found in normal peripheral blood, indicates that thoracic-duct cells synthesize a relatively larger quantity of immunoglobulins. This may be due to the fact that the majority of the thoracic-duct lymph samples were obtained from patients who were presumably more antigenically stimulated; only two of the samples may be regarded as normal in this respect. Another fact to be considered is that a large percentage of the lymphoid cells of the thoracic-duct lymph might recently have been sensitized, as many of them derive from the intestinal tissues and mesenteric nodes. These tissues can be considered to be subjected to antigenic stimuli more frequently than the other components of the lymphatic apparatus (Gowans and McGregor, 1963). Besides their origin, the morphology of the immunoglobulin-synthesizing cells of the thoracic-duct lymph also differs in that they include an appreciable number of large lymphocytes and cells of the plasma cell series. These cells are rare in normal peripheral blood.

The two infectious diseases studied, i.e. infectious mononucleosis and rubella, showed different patterns. In infectious mononucleosis during the first 10 days of illness, an increased synthesis of IgM was found as compared with normal blood samples containing equivalent numbers of lymphocytes. This increase was followed by a period of significant decrease of IgM synthesis. After about the second week of illness the synthesis was com-

parable to the results obtained with normal blood samples. This correlation between the pattern of IgM synthesis and the day of illness at the time of sampling suggests a biphasic course, but the number of the samples is too small to permit more than tentative conclusions. Support is found in the results of the immunofluorescent studies. During the first 10 days the staining for the three immunoglobulins was positive, but thereafter the number of IgM-positive cells decreased and the staining for IgG and IgA became negative.

The following interpretation may be considered. The atypical cells of the peripheral blood in infectious mononucleosis, a disease probably of viral origin and predominantly localized in the lymphoid tissues, were shown to be the result of active proliferation (Hale and Cooper, 1963). Furthermore, it is justified to assume that the infectious agent of this disease induces a primary antigenic stimulation known to be associated initially with the formation of predominantly IgM antibodies (Bauer and Stavitsky, 1961; LosPalluto, Miller, Dorward and Fink, 1962; Fink, Miller, Dorward and LosPalluto, 1962; Bauer, Mathies and Stavitsky, 1963; Uhr and Finkelstein, 1963; Svehag and Mandel, 1964a, b). Since the circulating lymphocytes derive from lymph nodes and the spleen, it may be postulated that the majority of the immunoglobulin synthesizing cells that reach the circulation during the first 10 days of illness have been very recently stimulated and are therefore mainly engaged in the synthesis of IgM. However, this does not explain the subsequent phase of impaired synthesis.

The pattern of immunoglobulin synthesis in chronic lymphatic leukaemia also warrants further discussion. A striking feature here was the almost complete absence of IgA and the labelling of the IgG and IgM line. The immunofluorescent staining also revealed no IgA-positive cells. The preferential labelling of the medium to high mobility part of the IgG line in the majority of the samples and the relative decrease of the low-mobility part cannot be satisfactorily explained by a simple impairment of production, for if this were the case a relative decrease of the high-mobility part of the IgG would be expected since this fraction, which comprises only a minor part of the total amount of the IgG, usually disappears in cases of hypogammaglobulinaemia (Hitzig, 1963). In this connection it may be relevant that the sera of two patients in fact showed a slight increase of high-mobility IgG and a third patient had a high-mobility IgG paraproteinaemia. Two alternative explanations may be considered. The leukaemic process may give rise to the proliferation of cells capable of synthesizing the observed pattern of immunoglobulins with a relative reduction of the number of cells synthesizing IgA and low-mobility IgG. Support for this hypothesis may be found in the occasional occurence in this disorder of paraproteins that may be being synthesized by closely related proliferating cells. The question of whether in chronic lymphatic leukaemia the specific pattern of immunoglobulins is synthesized by the leukaemic cells cannot be answered conclusively because in the immunofluorescent slides the positive cells could not be distinguished as either normal or pathological lymphocytes. The other possibility is that cells capable of synthesizing IgA and low-mobility IgG are selectively destroyed by the leukaemic process. The frequent occurence of hypogammaglobulinaemia in this disorder, which is apparently due to a reduction of the rate of synthesis (Jim, 1957; Hudson and Wilson, 1960; Fairley and Scott, 1961; Miller, Budinger and Karnofsky, 1962; Andersen, 1963), and the reduction or absence of the formation of specific antibodies after antigenic stimulation (Barr and Fairley, 1961; Fairley and Akers, 1962; Heath, Fairley and Malpas, 1964; Cone and Uhr, 1964) would support the latter explanation. The only other situation in which we found exclusive synthesis of IgG and IgM and the absence of IgA was in the human foetal spleen (van Furth, 1964; van Furth

et al., 1966c). Considering the similarity of the patterns of immunoglobulin formation *in vitro* between the foetal spleen and the peripheral blood lymphocytes in chronic lymphatic leukaemia, it is conceivable that the lymphatic apparatus in this disorder represents a more primitive stage.

Application of the immunofluorescent technique makes it possible to investigate the morphology of the cells that are in the process of synthesizing immunoglobulins. Although this staining was performed only on samples prior to incubation, it seems justifiable to assume that similar cells synthesize immunoglobulins *in vitro* because it has been shown that under our experimental conditions the main synthesis takes place during the initial 6–12 hours (van Furth, 1964, 1966). In blood samples from healthy donors the cells positive for IgG, IgA or IgM were predominantly medium-sized lymphocytes. Immunofluorescent studies of cytological preparations, made from animal and human tissues, also showed medium-sized lymphocytes which contain immunoglobulins or antibodies (Vasquez, 1961, 1964; Balfour, Cooper and Alpen, 1965; van Furth, Schuit and Hijmans, 1966b). In infectious mononucleosis, rubella and the thoracic-duct lymph, other cells, i.e. plasma cells and large lymphocytes, were also found to be positive. In all the samples, however, the majority of the lymphoid elements failed to show detectable amounts of immunoglobulin.

Electron-microscopic studies have shown circulating lymphocytes which possess structural components indicating that they are capable of synthesizing protein. Some large and medium-sized lymphocytes of the thoracic-duct lymph contain rough endoplasmic reticulum (Zucker-Franklin, 1963a,b; Marschesi and Gowans, 1964), and in infectious mononucleosis atypical cells in the peripheral blood may contain more endoplasmic reticulum than is found in the average lymphocyte (Paegle, 1961; Reinauer, 1961; Zucker-Franklin, 1963b). Small lymphocytes have only scanty cytoplasm, but some of these cells in the peripheral blood and thoracic-duct lymph have been shown to possess a small amount of rough endoplasmic reticulum (Zucker-Franklin, 1963b).

The *small lymphocytes*, which have been shown to contain only IgM, will be discussed in more detail. Although the small rim of weak fluorescence in the small lymphocytes could conceivably be due to the adsorbtion of IgM to their surface, there is considerable evidence contradicting this possibility. The fluorescence seen in these positively-stained cells differs from the rim of fluorescent immunoglobulins adsorbed by the cells in unwashed suspensions, and the positive cells in some cases demonstrate the presence of IgM in the form of cytoplasmatic granules, e.g. in infectious mononucleosis. The fact that the positive cells are limited to only one immunoglobulin and the variation in the number of positive small lymphocytes according to the origin of the investigated sample, are other arguments against adsorption. The complete absence of fluorescence after blocking procedures with unlabelled anti-IgM or after staining with an adsorbed anti-IgM conjugate which no longer reacted with IgM also point to a specific immunological interaction. It is therefore considered that the positive small lymphocytes actually contain IgM, albeit in very small amounts, which can nevertheless be detected because of the sensitivity of the immunofluorescent technique (Pressman, Yagi and Hiramoto, 1958).

Small lymphocytes, which contain antibodies or immunoglobulins, have also been demonstrated by others (Vasquez, 1961, 1964; Balfour *et al.*, 1965). Evidence has further been obtained that small lymphocytes are capable of synthesizing (Attardi, Cohen, Horibata and Lennox, 1959, 1964). However, these studies have not established whether the positive cells contain only IgM molecules.

At this point it is relevant to consider what immunological role is played by the IgM-positive small lymphocytes. As recently reviewed by Gowans and McGregor (1965), very suggestive evidence has been obtained from various investigations that the small lymphocyte may be involved in primary antibody response. The morphological sequence after primary antigenic stimulation will then be the development of the small lymphocytes into large dividing pyroninophylic cells and ultimately into plasma cells. The last step, however, has not been conclusively demonstrated. Studies on the kinetics of antibody formation have clearly demonstrated that in primary response the initially synthesized antibodies are predominantly IgM molecules. These antibodies can be detected soon after antigenic stimulation, and are followed after some delay by antibodies of the IgG type.

On the basis of these data the following hypothesis can be put forward: The positive fluorescence of the small lymphoctyes indicates their initial synthesis of IgM antibodies when these cells are engaged in primary response. This supposition is supported by the results of the immunofluorescent staining of various tissues. In the cell suspensions derived from lymphoid organs such as lymph nodes and spleen, which are usually engaged in the primary antibody response, a considerable number of IgM-positive small lymphocytes have been observed (van Furth, 1964; van Furth *et al.*, 1966b). In thoracic-duct lymph and normal peripheral blood samples, which contain lymphocytes derived from these organs, a smaller number of these positive small lymphocytes were present, but the bone marrow, which is considered not to participate in primary antibody response (Gengozian, Makinodan and Shekarchi, 1961; Langevoort, Asofsky, Jacobson, de Vries and Thorbecke, 1963; Friedman, 1964), showed no fluorescent small lymphocytes under normal conditions (van Furth, 1964; van Furth *et al.*, 1966b). The investigations concerning infectious mononucleosis provide additional support for this hypothesis because this infection may be regarded as inducing a primary antigenic stimulation; blood samples taken during the first 10 days of illness show a marked increase in the number of IgM-positive small lymphocytes as compared to normal blood samples. This also applies to the numerous positive small lymphocytes in rubella. The foetal spleen and foetal peripheral blood samples were also found to contain numerous IgM-positive small lymphocytes, for which the same explanation, i.e. predominantly primary response, may hold (van Furth *et al.*, 1966c).

Furthermore, it is tempting to speculate about a possible relationship between the delay in time before the appearance of IgG antibodies in primary response and the time interval involved in the transformation of the small lymphocytes into the larger pyroninophylic cells. Thus in the present studies fluorescence positive for IgG was present only in the medium-sized and large lymphocytes and cells of the plasma-cell series. The fluorescent medium-sized lymphocytes and plasma cells may then represent later stages of development of small lymphocytes. Further studies are required to investigate the sequence in which cells showing positive fluorescence for either IgM or IgG antibodies appear after primary antigenic stimulation in correlation with the concomitant production of antibodies.

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