myxoedematous state there may be a metabolic defect resulting in poor tissue-oxygen uptake.

It is unlikely that the exercise E.C.G. can be of help in determining whether a patient is liable to develop symptoms of cardiac ischaemia when treated for myxoedema.

We would like to thank Professor C. Wilson, Dr. W. Brigden, and Professor J. M. Ledingham for helpful discussion. This study formed part of an M.D. thesis (Cambridge) submitted by one of us (R. D. C.).

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# Impaired *in Vitro* Lymphocyte Transformation in Patients with Ataxia-telangiectasis

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Ataxia-telangiectasis is an autosomal recessive disorder characterized by gradually progressive cerebellar ataxia in childhood (Wells and Shy, 1957; Boder and Sedgwick, 1958) and telangiectasis secondary to venule dilatation (Louis-Bar, 1941; Williams *et al.*, 1960). These children often manifest impaired immune competence. They have an increased incidence of sinopulmonary infections (Gutmann and Lemli, 1963), undetectable or abnormal serum IgA ($\beta$-$_2$A) immunoglobulin levels (St. Thieffry *et al.*, 1961), diminished delayed hypersensitivity reactions, delayed and prolonged skin-homograft rejection, and occasionally peripheral lymphopenia (Fireman *et al.*, 1964; Young *et al.*, 1964; Eisen *et al.*, 1965). Absence or hypoplasia of thymic and lymphoid tissues, and an increased incidence of malignant lymphoma among these patients, have also been described (Peterson *et al.*, 1964).

The lymphocyte plays an important part in immune reactions (Gowans *et al.*, 1962). Small lymphocytes can be stimulated to transform *in vitro* to large blast cells, which may undergo mitoses. This response can be elicited both by non-specific stimulants such as phytohaemagglutinin (Nowell, 1960; Ling and Husband, 1964) and by specific antigens to which the cell donor has been previously exposed (Pearmain *et al.*, 1963; Elves *et al.*, 1963). Impaired lymphocyte transformation has been reported in patients with various immunological and lympho-proliferative disorders such as primary hypogammaglobulinaemia (Fudenberg and Hirschhorn, 1964; Ling and Soothill, 1964), Boeck's sarcoid and lymphomas (Hirschhorn *et al.*, 1964), and chronic lymphocytic leukaemia (Oppenheim *et al.*, 1965a, 1965b). We have tested the response to phytohaemagglutinin and various antigens of lymphocytes cultured from the peripheral blood of patients with ataxia-telangiectasis to characterize further their immunological deficiency.

## Patients Studied

The peripheral leucocytes of five untreated patients with characteristic ataxia-telangiectasis were repeatedly tested and compared with those obtained from 18 normal young adults. The patients ranged in age from 10 to 19. Two of them were sisters, and the others were unrelated. All had received childhood immunizations with diphtheria, pertussis, and tetanus and vaccinia vaccine without sequelae, and had had the common childhood viral infections without complications. Two of the patients had histories of frequent pulmonary and middle-ear infections. These had not recurred for three years prior to this study. One of the patients had a questionable allergic history of "rose fever" in the spring, and skin eruptions after ingesting chocolate.

Mediastinal tomography failed to reveal any thymic enlargement. The pharyngeal tonsillar tissue was decreased in all patients. None had peripheral lymphadenopathy. One of the patients was lymphopenic, with a mean of 810 peripheral lymphocytes per c.mm. Bone-marrow aspirations in the four patients examined were normal and contained plasma cells. All patients lacked detectable serum IgA immunoglobulin by immunoelectrophoretic and immunodiffusion techniques. The lymphopenic patient had hypogammaglobulinaemia by serum-protein electrophoresis. All patients had low normal iso-agglutinin and febrile agglutinin titres. They were all anergic to intradermal tests with blastomycin, histoplasmin, coccidioidin, mumps antigen, trichophyton, second-strength purified protein derivative, and trichinella extract. In the three subjects tested 2,4-dinitrochlorobenzene skin-sensitization elicited a normal reaction. They also rejected first-set, split-thickness, 8-mm. punch-biopsy skin homografts in normal fashion within 21 days. There was no evidence of malignant lymphomatous disease in any of the patients.

## Methods of Culturing Leucocytes

From each of the five patients 30–50 ml. of venous blood was obtained on three to six different occasions over a nine-

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months period. The *in vitro* peripheral leucocyte cultures were prepared by previously described methods (Oppenheim and Perry, 1965). The cell suspension of 1,000–2,000 white blood cells per c.mm. in a mixture of 30% autologous plasma and 70% minimal essential medium was divided into sets of 6-ml. aliquots. To the first aliquot 0.25 ml. isotonic saline was added, to the second 0.1 ml. phytohaemagglutinin-M, to the third 0.25 ml. vaccinia vaccine (1:10 diluted with saline), and to the fourth 0.25 ml. streptolysin-O (1:25 dilution in saline). On several occasions the responses of the patients' leucocytes to each other's and normal W.B.C. histocompatibility antigens were tested, the method of mixed leucocyte cultures (Bain *et al.*, 1964) being used.

To one set of cultures, from three of the patients, homologous thymic extract was added. Thymus was obtained from patients at the time of thoracotomy for repair of cardiac defects and stored at −10° C. The saline extract of thymus was prepared by means of a Potter-Elvehjem homogenizer at 4° C. Five grams of homogenized tissue was extracted with 20 ml. of isotonic saline for two hours, and 0.2 ml. of filtrate (after passage through an 0.8-$\mu$ Millipore filter) was added to each 6-ml. cell suspension.

In additional studies the patients' white blood cells were washed twice with medium and cultured in normal homologous plasma. Conversely, white blood cells obtained from normal donors were cultured in the patients' plasma. Other culture modifications were described in the text.

All cultures were incubated for five days at 37° C. Four hours prior to harvesting 0.2 ml. suspension of polystyrene particles (1:100 saline dilution, 1.3 $\mu$ diameter) and 0.33 $\mu$g. demecolcine (Colcemid) per ml. were added to each culture to aid in differentiating the non-phagocytic transformed lymphocytes from macrophages (Rabinowitz, 1964), and to elevate the mitotic index to more readily measurable levels (Nowell, 1960).

The cells were collected by gentle centrifugation, fixed with absolute methyl alcohol and glacial acetic acid, 9:1, pipetted on to slides, and stained with Giemsa. A 1,000-cell differential was done to determine the mitotic index, proportions of small and transformed lymphocytes, and macrophages present.

Tritiated thymidine (2 $\mu$c./ml., 0.360 c./mM specific activity) was added to two or three sets of cultures of four of the patients, and the same number of controls. Radioautographs taken with Kodak AR 10 were prepared in the usual fashion and developed after two weeks of exposure (Adamik—see ref.).

## Results

Usually more than 50% of the cultured lymphocytes from patients with ataxia-telangiectasis manifested evidence of cell damage. This consisted of ruptured nuclear membranes, fragmentation, excessive vacuolization, karyolysis, karyorrhexis, hyalinization, or pyknosis. Generally, the cultural normal lymphocytes manifested less than 20% cell damage. The proportion of surviving macrophages was also higher, but not significantly so, in the patients than in the normal cultures (Table I).

The median proportion of lymphocytes that transformed was 34% in the patients' phytohaemagglutinin-stimulated cultures. This was significantly less than the 88% seen in those of the normal subjects. Similarly, when the antigens, streptolysin-O, and vaccinia were used only 3% and 0% of the patients' lymphocytes transformed, compared with normal responses of 26% and 3% respectively. The patient–patient and patient–normal leucocyte mixtures manifested significantly less lymphocyte transformation than the normal–normal mixes. In general both the mitotic indices and proportions of mononuclear cells labelling with tritiated thymidine were much lower in the patients' than in the controls' stimulated cultures. The proportions of damaged lymphocytes were usually inversely proportional to the above manifestations of lymphocyte proliferation.

Duplicate cultures of the patients' white blood cells obtained at a given time manifested similar responses. However, the proportions of transformed lymphocytes obtained at different times varied over a wide range from none to normal in response to phytohaemagglutinin and antigens (Fig. 1). The lymphocyte

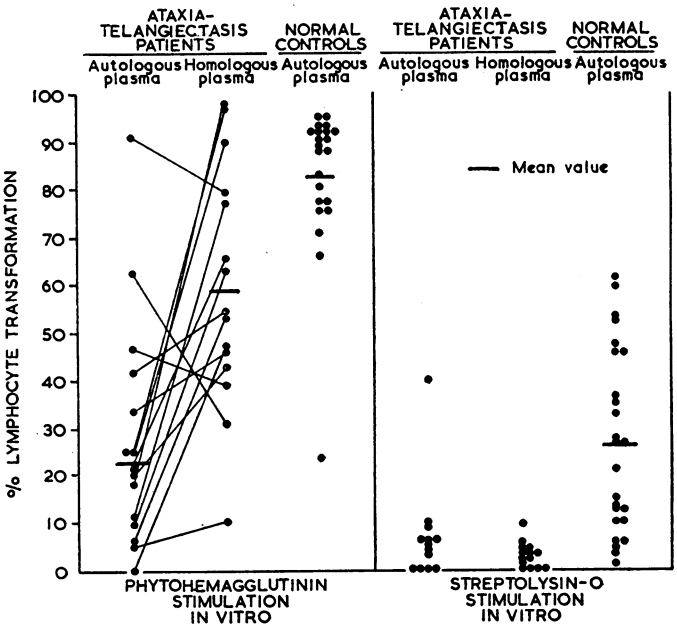


FIG. 1.—Connected symbols refer to paired leucocyte cultures performed simultaneously and differing only in the plasma used for medium supplementation. The three high transformations with phytohaemagglutinin noted in patients' autologous plasma are referred to in the text. Paired cultures testing the effects of homologous plasma on transformation response to streptolysin-O are not similarly connected because of the general lack of response in these ataxia-telangiectasis cultures.

TABLE I.—*Comparison of Ataxia-telangiectasis and Normal Peripheral Leucocyte Cultures Median Responses to Phytohaemagglutinin-M and Antigens*

| Stimulant | Subjects | No. of Exp. | % Macrophages | Mitotic Index | % Transformed Lymphocytes | % Mononuclear Cells Labelling with TdRH³ | Chi² Test of Normal *v.* Patients' Lymphocyte Transformation (P Value) |
|---|---|---|---|---|---|---|---|
| None | A.-T. patients | 20 | 48 (0–70) | 0 | 0 (0–1) | 0 | 0·60 |
| | Controls | 19 | 19 (5–83) | 0 | 0 (0–2) | 0 (0–1) | |
| P.H.A.-M | A.-T. patients | 30 | 6 (0–55) | 0 (0–1) | 34 (0–91) | 10 (0·4–28) | 0·001 |
| | Controls | 18 | 1 (0–9) | 8 (0–40) | 88 (23–93) | 12 (7–21) | |
| SLO | A.-T. patients | 25 | 36 (0–58) | 0 (0–1) | 3 (0–40) | 0 (0–23) | 0·001 |
| | Controls | 21 | 13 (2–50) | 11 (0–54) | 26 (1–61) | 15 (5–25) | |
| Vaccinia | A.-T. patients | 21 | 5 (0–90) | 0 (0–1) | 0 (0–6) | 1 (0–4) | 0·01 |
| | Controls | 11 | 5 (0–19) | 0 (0–4) | 3 (0–17) | 3 (0–10) | |
| W.B.C. mixes | Patient–Patient | 3 | 56 (8–57) | 0 (0–1) | 1 (1–4) | — | 0·005 |
| | Patient–Normal | 4 | 35 (4–54) | 0 | 1 (0–3) | — | |
| | Normal–Normal | 8 | 10 (3–23) | 7 (1–32) | 14 (4–35) | — | |

Numbers in parentheses indicate range. A.-T. = Ataxia-telangiectasis. P.H.A. = Phytohaemagglutinin. SLO = Streptolysin-O. TdRH³ = Tritiated thymidine.

transformation of three of the patients was maximal at the time of their skin-homograft rejections.

The patients' transformed lymphocytes were usually much smaller than normal (Fig. 2). The size of the responding cells also varied directly with the proportions of cells responding, and approached normal in those cultures which manifested normal lymphocyte proliferation.

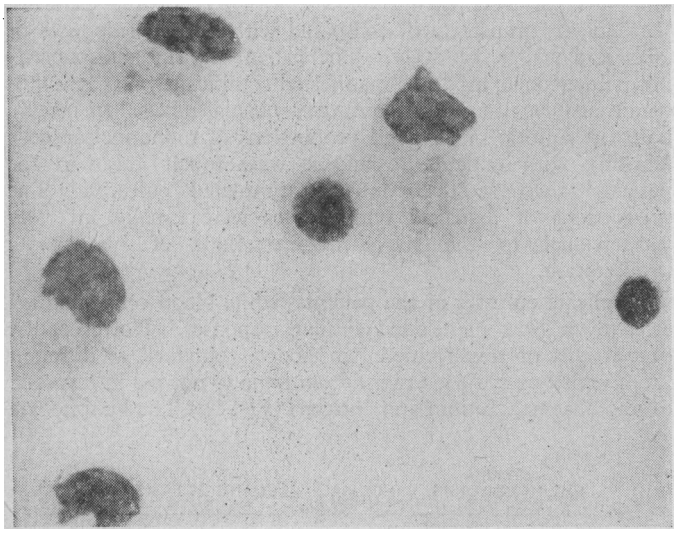

FIG. 2.—Phytohaemagglutinin-stimulated ataxia-telangiectasis culture in autologous plasma (×1,030). Four macrophages, a small transformed blast cell, and a dead lymphocyte are seen.

Attempts to improve the patients' lymphocyte growth by culturing their cells in enriched media (TC135, kindly supplied by Dr. K. Sanford) failed. The additions of 100 mg. of fructose per 100 ml. of glucose-free media, or insulin (low zinc) 0.7 mg./ml. medium, did not alter the growth of ataxia-telangiectasis cells *in vitro*. Reconstitution of autologous plasma with homologous IgA to a final concentration of 50 mg./100 ml. did not improve cell growth. The addition of homologous thymic extract also failed to restore to normal *in vitro* growth of the patients' leucocytes, and had no significant effect on cultured normal leucocytes.

TABLE II.—*Effect of Culturing the A.-T. Patients' Peripheral Leucocytes with Plasma and Thymic Extract, and the Median Lymphocyte Transformation of Normal Leucocytes Cultured with the Patients' Plasma*

| Stimulant | Subjects | Cultured with | No. of Experiments | Mitotic Index | Percentage Transformed Lymphocytes | Proportion of Mononuclear Cells Labelling with TdRH³ |
|---|---|---|---|---|---|---|
| P.H.A.-M | A-T | Autologous plasma | 22 | 0 (0–1) | 15 (0–91) | 2 (0·4–23) |
| „ | „ | Homologous plasma | 15 | 0 (0–1) | 57 (31–79) | 18 (10–28) |
| „ | „ | Thymic extract | 3 | 1 (0–1) | 35 (28–89) | — |
| SLO | A-T | Autologous plasma | 18 | 0 (0–1) | 3 (0–40) | 3 (0–23) |
| „ | „ | Homologous plasma | 14 | 0 | 3 (0–9) | — |
| „ | „ | Thymic extract | 3 | 0 | 1 (0–0) | — |
| Vaccinia | A-T | Autologous plasma | 13 | 0 (0–0) | 1 (0–6·0) | 2 (0–4·0) |
| „ | „ | Homologous plasma | 6 | 0 | 0 (0–2·0) | — |
| „ | „ | Thymic extract | 2 | 0 | 0·5 (0–1·0) | — |
| P.H.A.-M | Control | Autologous plasma | 5 | — | 92 (92–95) | — |
| „ | „ | A.-T. plasma | 5 | — | 91 (81–97) | — |
| SLO | Control | Autologous plasma | 5 | — | 19 (3–46) | — |
| | „ | A.-T. plasma | 5 | — | 12 (0–48) | — |

Numbers in parentheses indicate range.

However, when the ataxia-telangiectasis patients' cells were cultured with phytohaemagglutinin in homologous rather than autologous plasma there was a significant increase at their lymphocyte transformation to 57% ($P=0.005$ by the $chi^2$ method) (Fig. 1, Table II). Both the proportions and size (Fig. 3) of the transforming lymphocytes were considerably improved in three of six paired cultures when frozen stored normal plasma was used, and in nine out of nine paired cultures cultured with fresh normal plasma or calf serum. The proportions of damaged lymphocytes also were decreased by the presence of homologous plasma in the unstimulated as well as the stimulated cultures. The response to streptolysin-O was not significantly or consistently improved by homologous plasma. The growth of normal human leucocytes was not inhibited by culturing them in the ataxia-telangiectasis patients' plasma (Table II).

## Discussion

In attempting chromosome preparations of five patients with ataxia-telangiectasis, Eisen *et al.* (1965) had difficulty in stimulating leucocyte growth in short-term cultures with phytohaemagglutinin. Our cultured leucocytes from five ataxia-telangiectasis patients also failed to grow well, both with phytohaemagglutinin and antigenic stimulants. The cultures consistently manifested considerably more damaged lymphocytes than normal leucocyte cultures. The proportion of surviving macrophages was also generally higher in the patients' cultures. Increased proportions of macrophages have also been found when normal cells were cultured with endotoxin (Oppenheim and Perry, 1965), horse anti-human-cell antisera (Blaylock *et al.*, 1966), and in leucocyte cultures from patients with Hodgkin's disease (Hersh and Oppenheim, 1965). As absolute cell counts of macrophages were not done in the present study, nor in those referred to, the increased percentage of macrophages may be due only to relatively greater macrophage survival as compared with lymphocytes in the ataxia-telangiectasis leucocyte cultures.

The patients' *in vitro* lymphocyte proliferation in response to phytohaemagglutinin, antigens, and homologous white blood cells, as indicated by their lymphocyte transformation, by mitotic indices, and usually by their tritiated thymidine uptake, was significantly less than comparably stimulated normal leucocytes. Also, those lymphocytes responding to phytohaemagglutinin generally appeared smaller in size than normal transformed lymphocytes. The maximum response in three of these patients' cultures to both phytohaemagglutinin and antigens was associated with a skin-homograft rejection. The

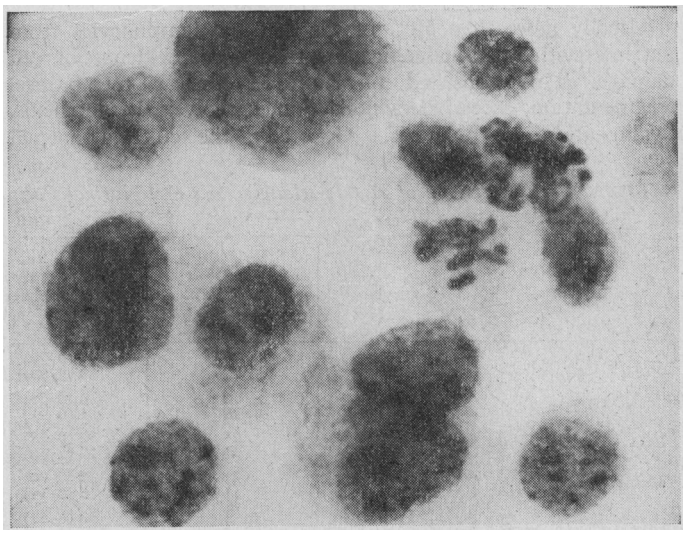

FIG. 3.—Phytohaemagglutinin-stimulated ataxia-telangiectasis culture in homologous fresh plasma (×1,030). Paired culture of Fig. 2. Large transformed blast cells and mitotic figures are noted.

studies of Oppenheim *et al.* (1965a) and Moynihan *et al.* (1965) suggest that the proportions of immunocompetent lymphocytes may be temporarily increased in the peripheral circulation of normal humans and monkeys at such a time.

The five ataxia-telangiectasis patients studied here manifested both a selective IgA immunoglobulin deficiency and a failure to react to a number of fungal, viral, parasitic, and mycobacterial antigens. However, they rejected skin homografts and could be sensitized to 2,4-dinitrochlorobenzene in a normal fashion. Therefore they were only partially defective immunologically, and, similarly, their *in vitro* lymphocyte transformation was only variably and partially impaired.

The aetiology of the impaired leucocyte growth in ataxia-telangiectasis patients was investigated by varying the *in vitro* environment. Only when the patients' cells were cultured in medium with homologous instead of autologous plasma did survival of lymphocytes improve. Both the size and proportions of transforming lymphocytes stimulated with phytohaemagglutinin significantly improved and often approached normal in those cultures employing homologous plasma. However, the response to streptolysin-O and other antigens was not significantly influenced by homologous plasma. Conversely, normal cells were not adversely affected when cultured in the patients' fresh plasma. These findings argue against the presence of a toxic factor in the patients' plasma, but suggest that there may be a partial deficiency of factor(s) necessary for cell growth. The data also suggest that the decreased survival and impaired proliferation of the viable lymphocytes in ataxia-telangiectasis leucocyte cultures may not be due to an intrinsic abnormality of their lymphocytes, but be secondary to some non-lethal genetic deficiency, which can be rectified to some extent by factors present in normal plasma. The identification of these plasma factors, their relationship to clinically expressed immune-incompetence, and the impaired lymphocyte transformation noted in other disorders require further study.

## Summary

The leucocytes of five patients with typical ataxia-telangiectasis, IgA immunoglobulin deficiency, and partial anergy were repeatedly cultured with phytohaemagglutinin-M and antigens such as streptolysin-O, vaccinia vaccine, and homologous leucocytes. The cultures manifested more damaged lymphocytes and higher macrophage survival than did 18 normal controls. Lymphocyte proliferation, as measured by transformation, mitotic indices, and tritiated thymidine uptake in response to both phytohaemagglutinin and antigens, was significantly impaired. The diminished numbers of lymphocytes which transformed were generally smaller in size than comparably stimulated normal cells. Lymphocyte survival and both the proportions and the size of transformed lymphocytes responding to phytohaemagglutinin were increased when homologous instead of autologous plasma was added to the medium.

We would like to express our gratitude to Drs. E. Hersh and B. Leventhal for their suggestions; to Dr. W. K. Engel, Medical Neurology Branch, National Institute of Neurological Disease and Blindness, for providing the opportunity to study these patients; and to Mr. J. Cirvello for technical assistance.

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# Effect of Frusemide on Carbohydrate Metabolism, Blood-pressure, and other Modalities: a Comparison with Chlorothiazide

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The benzothiadiazine drugs have recently been incriminated as diabetogenic (Wilkins, 1959; Goldner *et al.*, 1960; Sugar, 1961; Zatuchni and Kordasz, 1961; Wilson, 1963; Samaan *et al.*, 1963; Rapoport and Hurd, 1964; Wolff and Parmley, 1964), and the onset of asymptomatic chemical diabetes has often been reported during their use. The related diuretic, chlorthalidone, has also been implicated. The present trial was undertaken to see whether the newer diuretic frusemide (Lasix) had any effect on carbohydrate metabolism and to compare it with chlorothiazide in a group of selected moderately hypertensive outpatients. In some ways this trial resembles that of Shapiro *et al.* (1961), who examined the effect of chlorothiazide on the glucose tolerance and the intravenous tolbutamide tolerance in 30 hypertensive subjects.

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## Patients and Methods

Twenty new patients who attended the hypertension clinic between the ages of 40 and 70 were chosen on the basis that they appeared suitable for the trial by reason of intelligence,