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REVIEW ARTICLE

CURRENT CLINICAL APPLICATIONS OF LYMPHOCYTE TISSUE CULTURE

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INTRODUCTION

Unde etiam vulgare Graeciae dictum
'semper aliquid novi Africam adferre'
Pliny

SELDOM a week goes by without someone documenting a new discovery in lymphocyte tissue culture which has potential application to clinical problems. It is very much with this super-abundance of data in mind that the present review has been prepared in an attempt to give the busy practising clinician a bird's-eye view of the current literature, so that on rounds he might be prompted to consider the question: 'Is there, possibly, a lymphocyte tissue culture procedure which might help with this patient's diagnosis or prognosis?' It must be said at the outset that the authors have not tried to be fully comprehensive in the computerized connotation of that phrase, rather have they been selective in two ways; firstly a number of review articles and books (Table I), notably that by Noel Ling (68), are available to supply the background to the subject, so that attention has been concentrated on recent publications. Secondly they have selected for consideration the main growing points that could be expected to be of interest to the clinician and those highlights which may prove to have a biological significance above and beyond their immediate potential in clinical research. In attempting to paint the broad picture a certain price has had to be paid in terms of leniency with regard to the criticism that could have been directed at some of the culture techniques, and toward some of the methods of quantification. The reviewers' motive in overlooking these technical points for the time being, has been a desire to plant in

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TABLE I

BOOKS AND ARTICLES PROVIDING BACKGROUND TO LYMPHOCYTE TISSUE CULTURE

Author and Reference	Year	Topic covered
Ambs (2)	1969	Clinical aspects
Bain (6)	1968	Mixed leucocyte cultures
Cooper and Amiel (16)	1965	General
Coulson (18)	1967	History
Harris (41)	1970	Recent research
Holm and Perlmann (48)	1969	Cytotoxicity
Ling (68)	1968	General
McIntyre (83)	1969	Recent research
Meuwissen, Stutman and Good (87)	1969	General
Naspitz and Richter (90)	1968	Phytohaemagglutinin
Oppenheim (93)	1968	Immunology
Rieke (104)	1969	General
Weber (132)	1969	Phytohaemagglutinin

the clinician's mind the principle, that when assessing a patient's immunological capabilities he should bracket together as related parameters, lymphocyte tissue culture and any measurement of the patient's serological responsiveness. In addition the physician may come to look on the patient's lymphocytes *in vitro* as readily attainable living biopsy material, and as such a potential mine of information about the behaviour of the patient's somatic cells. The day may soon come when request forms are available on the wards on which one can tick off 'lymphocyte function tests', as one can at present 'liver' or 'respiratory function tests'. The variety of clinical conditions capable of examination by lymphocyte tissue culture and the ingenuity of some of the approaches will serve to emphasize how wide ranging is the applicability of this technique, and how firmly it is becoming established as a clinical laboratory test.

IMMUNOLOGY

It may eventually prove to be an oversimplification, nevertheless it is at present a useful clinical concept, to think of the *in vitro* immunological responsiveness of blood lymphocytes as correlating with the thymus-dependent population of small lymphocytes in the blood sample taken. Since the delayed hypersensitivity status of the lymphocyte donor is also related to the thymus-dependent population, the lymphocyte transformation test is, as could be anticipated, a good *in vitro* measure of the potential *in vivo* delayed hypersensitivity response; that is the response more rationally designated Type IV by Coombs and Gell (15). The relationship between the *in vitro* lymphocyte transformation test and the circulating thymus dependent lymphocytes rests on two sound lines of evidence which can be conveniently summarized

in tabular form. Firstly, a number of authors have demonstrated a direct correlation between donor-delayed hypersensitivity status and the response of his lymphocytes *in vitro* (Table II). Secondly, it has been shown in humans with congenital defects in their immunological apparatus that when delayed hypersensitivity responsiveness is impaired *in vivo*, this is mirrored by much reduced *in vitro* responsiveness to antigenic challenge on the part of the lymphocytes. Conversely if immunoglobulin production is defective and *in vivo* immunological responses mediated by antibodies are impaired (that is Types I, II and III of Coombs' and Gell's classification), lymphocyte transformation *in vitro* is not affected provided cellular immunity *in vivo* can still be demonstrated in the patient (Table III).

Accumulating data gleaned from recent publications tends to support the broad view that equates lymphocyte responsiveness to antigenic stimulus *in vitro*, with the *in vivo* thymus dependent population. Thus for example Kisken and Swenson (57), having

TABLE II

AUTHORS WHO HAVE DEMONSTRATED A CORRELATION BETWEEN DELAYED HYPERSENSITIVITY (TYPE IV RESPONSE), AND *in vitro* LYMPHOCYTE RESPONSE ON THE PART OF HUMAN SUBJECTS

Authors	Antigen
Coulson and Chalmers (20)	tuberculin
Kelley, Stanfield, Dukes and Parsons (54)	coccidioidin
Kerby (56)	tuberculin
Levan, Korn and Pineda (65)	coccidioidin
McFarland and Heilman (81)	tuberculin
Newberry, Chandler, Chin and Kirkpatrick (92)	histoplasmin
Shannon, Johnson, Rosen and Austen (118)	candidin
Zweiman, Pappagianis, Maibach and Hildreth (137)	coccidioidin

TABLE III

CONTRAST BETWEEN THE EFFECT OF IMPAIRED CELLULAR AND IMPAIRED HUMORAL IMMUNITY, ON THE *in vitro* RESPONSE TO ANTIGEN

Disease	Effect on the patient's immunity		Response to antigenic stimulation *in vitro*
	Humoral	Cellular	
Bruton-type non-lymphopenic hypogammaglobulinaemia	Impaired	Normal	Normal (36)
Ataxia telangiectasia	Increased IgG and IgM but absent IgA	Impaired	Impaired (37)
Wiskott-Aldrich Syndrome	Impaired	Impaired	Impaired (95)

established that thymectomy leads to prolongation of skin graft survival in dogs, were able to show that *in vitro* the lymphocytes from four chronically thymectomized dogs were unable to respond in mixed lymphocyte culture to unrelated lymphocytes. Working with human lymphocytes, Scheurlen, Pappas and Wegener (116) re-investigated the well-established model of tuberculin stimulation and confirmed the significant correlation between the *in vitro* response and the skin test. Both healthy tuberculin-positive donors and tuberculous patients fitted into the pattern; a mean of 10 per cent transformed cells after 5 days paralleling a skin reaction to 1 : 100 000 tuberculin, and 2.5 per cent transformed cells correlating with a response only to the much stronger 1 : 100 dose. Sørensen, Andersen and Giese (120) similarly showed that one donor with a violent skin reaction to 0.02 μg of purified tuberculin had an *in vitro* tritiated thymidine uptake of 23 000 cpm after 72 hours *in vitro*, while the other donor who had experienced only reddening and induration over the test site had an uptake of only 7000 cpm. A similar trend was seen with coccidioidin (137); subjects with a strongly positive skin reaction to 1 : 100 coccidioidin had a much increased tritiated thymidine uptake after 6 days of culture, while lymphocytes from weakly positive or from negative subjects did not respond *in vitro*. The well-established antigen dose–response phenomenon *in vitro* was also noted with this antigenic system. The consistency of the findings in recent years has given a lot of weight to Oppenheim's pointed comment that, 'as far as I am concerned we should redefine delayed hypersensitivity in terms of the inhibition of macrophage migration and positive lymphocyte transformation rather than in terms of positive delayed skin tests only' (93). The work of Ricci, Romagnani, Passaleva and Biliotti (102), demonstrated again that the *in vitro* transformation of guinea-pig lymphocytes paralleled the appearance of delayed skin reactions to the antigen used, but they found by examining at weekly intervals that inhibition of peritoneal cell migration was delayed until the *in vivo* delayed hypersensitivity response was considerable. Interestingly, when they investigated the guinea-pigs' serology they found no relationship between the *in vitro* phenomena and the presence in the sera of anti-purified-protein-derivative-haemagglutinating antibodies.

The work of Kačaki, Bullock and Vaughan (52) provides an example of failure to obtain *in vitro* stimulation with an antigen to which *in vivo* there is circulating antibody. After the addition of human fraction II and whole gamma globulin to the lymphocytes of rheumatoid-arthritis patients the transformed cells produced did not exceed those found in control cultures. This is in keeping

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with the work of earlier authors who have shown that there is no delayed hypersensitivity reaction *in vivo* to gamma globulin in these patients. Significantly Kačaki *et al.* (52) reported that the lymphocytes were washed three times in TC 199 medium before culture. That this may be an important point is shown by the work of Möller (88) where antigen—antibody complexes were found to stimulate DNA synthesis in normal human lymphocytes whereas antibody alone or antigen alone, had a negligible effect. The antibodies were produced in various species against a variety of antigens, but the species origin did not influence the results nor did the addition of fresh complement. Similarly Bloch-Shtacher, Hirschhorn and Uhr (10), showed that lymphocytes from donors not sensitized to a Salmonella antigen or to bovine serum albumin did not respond to the antigen alone *in vitro*, but did respond to aggregates of antigen and the appropriate antibody. They suggested that stimulation occurred as a result of damage to the lymphocyte membrane by the immune complexes. From a clinical viewpoint the findings of Gurvich and Svet-Moldavskaya (40), relating the extent of *in vitro* transformation to the time of previous vaccination against smallpox are of interest. They were unable to find any distinct correlation of virus-neutralizing antibody titre and transformation, but there was a correlation with the time lapse from the previous vaccination. Lymphocytes from donors immunized 6 years previously produced between 6 per cent and 9.8 per cent transformed cells whereas those immunized 20 or more years previously produced less than 3 per cent transformed cells. Antibodies did not seem to play any part in the *in vitro* reactions studied by Kelley, Stanfield, Dukes and Parsons (55). They found a clear difference between the *in vitro* responses of donors sensitive to coccidioidin and those not so sensitized, in terms of transformed cell production, and further, following skin testing there was an increase in the *in vitro* response; but antibodies could not be found by agar-gel immunodiffusion in either positive or negative donors.

Turning from stimulation with antigens *in vitro* to the non-specific effect of phytomitogens, of which phytohaemagglutinin has been the most thoroughly investigated, the current interpretation of their action is that they stimulate the transformation of that population of small lymphocytes in a culture which is thymus-dependent. It is not yet known whether all the thymus-derived cells are triggered, or whether there may be a refractory residual population of thymus-derived cells which with the bone-marrow-derived cells, make up the 30 per cent or so unresponsive small lymphocytes in a culture. The strongest line of evidence to support the view that phytohaemagglutinin triggers thymus-

derived cells comes from the work that has been carried out with cells from patients with congenital lymphoid diseases and is shown summarized in Table IV. Briefly when there is impaired cellular immunity (Type IV) *in vivo*, the *in vitro* phytohaemagglutinin response is absent; conversely those congenital diseases that result in impaired humoral immunity leave the patient with a normal phytohaemagglutinin response. This means that by using the drug phytohaemagglutinin it is possible to assess the number of thymus-dependent small lymphocytes present in a patient's blood sample. A point must, however, be made at this stage that emerges later in the discussion of general medical conditions, and that is that some disease states are accompanied by the production of plasma factors that inhibit *in vitro* responses. Hence a depressed response may mean either a reduction in the number of thymus-dependent lymphocytes, or the presence of such plasma factors. Having made allowances for this complication, the culture of a patient's lymphocytes amounts effectively to a living biopsy of his cellular immune system, a unique type of biopsy which permits a detailed examination of its behaviour and its dynamics over a relatively prolonged period of time.

Perhaps one of the most pertinent applications of lymphocyte tissue-culture to problems in clinical practice is that related to its use in attempts to clarify drug sensitivity reactions. In his review Levene (66), pointed out that many of the patients investigated had reactions which at best could only be alleged to be due to the drugs given and further that the antigens used *in vitro* and the culture quantitation were often not beyond serious criticism; particularly telling is his remark, 'One recalls protracted discussions on ward rounds as to whether a particular eruption was the result of penicillin therapy or of the underlying condition for which it was given.' Many of the drug reactions investigated were of the immediate type (Coombs Type I), and would not be expected to be matched by cellular sensitivity *in vitro* unless there were a Type IV component to the *in vivo* drug reaction. Levene was able to list a conflicting series of results published over the years with penicillin-sensitive patients, and to draw attention to the divergence of techniques used and to the fact that in some series the clinical diagnosis and the *in vitro* findings were so remarkably accurately correlated as to be almost suspicious. He concluded, as would many lymphocyte tissue-culture workers, by urging caution before accepting lymphocyte transformation as a test for drug allergy. Having made this point, however, it must be recorded that there are those who have lately reported a correlation between drug allergy and *in vitro* response, notably Kochman, Charreire. de Montreynaud and de Montreynaud (60). These

TABLE IV

RESPONSE TO PHYTOHAEMAGGLUTININ *in vitro* OF THE LYMPHOCYTES FROM PATIENTS WITH VARIOUS CONGENITAL DISEASES, ILLUSTRATING THE POINT THAT PHYTOHAEMAGGLUTININ TRIGGERS OFF THE THYMUS-DEPENDENT POPULATION

Congenital illness	Effect on immunity		Response to phytohaem-agglutinin	Reference
	Humoral	Cellular		
Thymic aplasia	Normal	Absent	Absent	Lischner, Punnett and DiGeorge (69); August, Rosen, Filler, Janeway, Markowski and Kay (4)
Swiss-type lymphopenic hypogammaglobulinaemia .. (Thymic dysplasia)	Impaired	Impaired	Absent	Meuwissen, Bach, Hong and Good (86); Gotoff (37)
Ataxia telangiectasia	Increased IgG and IgM, but deficient IgA	Impaired	Impaired	Oppenheim, Barlow, Waldman and Block (94); Gotoff (37)
Bruton-type non-lymphopenic hypogammaglobulinaemia	Impaired	Normal	Normal	Cooperband, Rosen and Kibrick (17); Bach, Meuwissen, Albertini and Good (5); Gotoff (37); Marshall, Cope, Soothill and Dudgeon (77)

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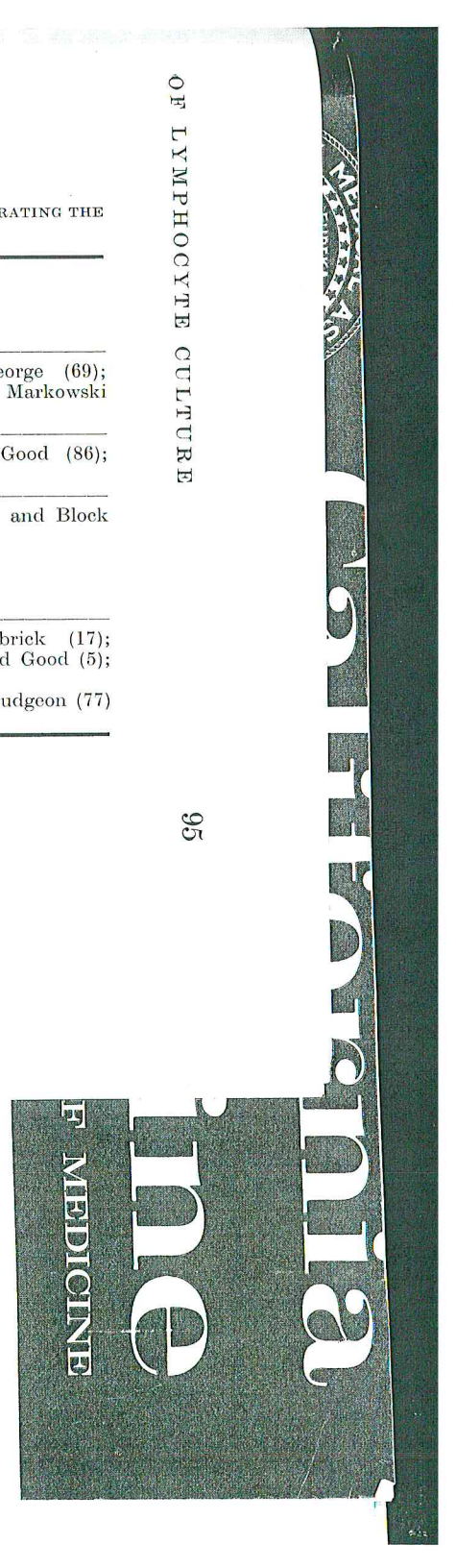

workers found, for example, that twenty-nine out of thirty-six patients with penicillin allergy responded *in vitro*, and similar figures were obtained with aspirin- and iodine-sensitive patients. Positive reports have also come from Reichenberger and Heitmann (100) employing lymphocytes from patients allergic to ampicillin and tetracycline, and from Savel, Madison and Meeker (114) using cells from two patients apparently sensitive to the mestranol in oral contraceptive pills. Although nickel-sensitivity is not strictly a drug allergy, it is convenient at this stage to consider the work of Macleod, Hutchinson and Raffle (72); twelve patients with unequivocal type IV patch-test reactions to 2.5 per cent nickel sulphate were found to have a thymidine uptake substantially above controls when challenged with 10^{-4} mEq Ni/ml *in vitro*. In nickel hypersensitivity the protein conjugate is unknown but the wealth of protein and polypeptides in the culture medium offer a multitude of possible carriers for the nickel hapten.

A more restricted application of lymphocyte tissue-culture, relating purely to its clinical immunological aspect, is seen in the monitoring of immunosuppression. The onset of rejection episodes and the effects of therapy on the patients' cellular immunity, have been followed *in vitro* by Tennenbaum, St Pierre and Cerilli (122). They reported, as did Hersh, Butler, Rossen and Morgan (45), that an effective way of detecting increased *in vivo* immunological activity against a renal allograft was to pick up an increase in the rate of spontaneous transformation of peripheral blood lymphocytes in culture. It was also found that a depression of phytohaemagglutinin stimulated transformation occurred when immunosuppression therapy became adequate. By way of contradiction, Heine, Stobbe, Klatt, Apostoloff and Dutz (44), found that the depression of phytohaemagglutinin response was only insignificantly lower in a group of thirty patients being treated with 6-mercaptopurine or azathioprine when compared to a group of fifty-four controls. They concluded that the effect of long-term treatment with purine antimetabolites in doses of 1.5 to 2 mg/kg body weight/day, could not be demonstrated by lymphocyte tissue-culture. Very possibly the doses of immunosuppressive drugs used by Tennenbaum, St Pierre and Cerilli (122) were greater than this, but it is not recorded in their paper. Another limited immunological application of lymphocyte tissue culture is in the diagnosis of syphilis from the response of a patient's lymphocytes to Reiter antigen or cardiolipin *in vitro*. Exposure to either of these antigens resulted in transformed cell production and increased thymidine uptake if the patient had a positive T.P.I. (119).

In this section the emphasis in discussing lymphocyte response to antigen *in vitro* has been very much to equate it to the type IV reaction *in vivo* (delayed hypersensitivity), and although this may eventually prove to be an oversimplification it is at the present time a most valuable clinical rule of thumb. Lymphocyte tissue culture provides what has been a long-felt need for an *in vitro* test of the type IV reaction particularly in that it permits greater standardization than skin testing, does not subject the patient to the trauma of a skin lesion with the attendant dangers of actually making the patient allergic, of precipitating an acute attack, or of worsening the patient's hypersensitive status. For these reasons it is more desirable to have the patient's lymphocytes *in vitro* as a living biopsy where their behaviour can be observed directly and quantitated.

In addition to being able to measure the patient's specific responsiveness to a given antigen experienced in the past, lymphocyte culture allows the clinician to assess the state of the thymus-dependent blood lymphocytes at the time of testing; in a manner of speaking it permits him to measure the thymus-dependent reserve. This is possible by virtue of the action of phytohaemagglutinin, as various experiments of nature have demonstrated that this drug triggers off only the thymus-dependent small lymphocyte population *in vitro*; in those disease states where such cells are depleted or absent this is mirrored by an appropriately reduced *in vitro* response. Hence phytohaemagglutinin culture joins the battery of tests available to the clinician which enable him to assess the patient's endocrine functions, and in particular it joins that group of tests used to measure the endocrine functioning of branchial pouch derivatives.

It must be obvious already from this brief look at the direct clinical applications of lymphocyte tissue culture immunology that dermatologists and pharmacologists could find considerable application for the technique. There is much on the technical side that needs standardization, and the repeatability of the results needs careful scrutiny, but when this is achieved lymphocyte culture should have great potential in the assessment of patients' sensitivity to drugs. In addition, subject to the same strictures on the practical side, lymphocyte culture could be valuable for monitoring the degree of immunosuppression in a patient.

RELEASE OF FACTORS

Increasingly in recent publications attention is being paid to the release of biologically effective factors, or lymphokines as they are better termed (26), by stimulated small lymphocytes or transformed cells. The macrophage inhibiting factor (molecular

weight about 60 000) is a good example of such a factor, being produced when sensitized lymphocytes come into contact with immunizing antigen, and resulting in the inhibition of movement of macrophages which it appears to reach by diffusion through the surrounding medium. Upon intradermal injection macrophage inhibiting factor has been shown to result in erythema and induration which become maximal at around 12 hours (8). A very similar factor has been described by Pick, Krejči, Čech and Turk (99); when they challenged lymphocytes from presensitized guinea-pigs with tuberculin or with phytohaemagglutinin, the culture supernatants were capable of provoking intense inflammatory reactions in the skin of normal guinea-pigs. The peak response in terms of erythema and induration occurred within 3–6 hours, and the population of cells that appeared was a mixed neutrophil polymorph and mononuclear infiltrate. Both Thor *et al.* (126), and Rocklin, Meyers and David (105) have demonstrated that when *human* blood lymphocytes are cultured with specific antigen they produce a soluble factor which can be shown to inhibit the migration of guinea-pig peritoneal exudate cells. In addition, the latter group have reported that their technique has been used successfully to detect type IV delayed hypersensitivity to glomerulonephritis antigens, and also in the study of certain neurological diseases, Hodgkin's disease and sarcoidosis. There is also some evidence to suggest that guinea-pig lymph-node lymphocytes may produce a chemotactic factor (129). Such cells, taken from animals previously sensitized to chlorobenzoyl chloride conjugated to bovine gamma globulin, when exposed to the same antigen *in vitro* liberated a factor which caused an active inward migration of rabbit peritoneal exudate cells. Such a factor produced *in vivo* by a few sensitized cells might perhaps explain why the preponderance of mononuclear cells at the site of a localized antigen injection in a type IV skin reaction are not specifically sensitized to the antigen employed.

In 1967 Kasakura and Lowenstein (53) described a factor released by stimulated lymphocytes in human mixed lymphocyte cultures which stimulates other small lymphocytes to transform non-specifically, a so-called blastogenic factor. Similarly Maini, Bryceson, Wolstencroft and Dumonde (73) have presented strongly suggestive evidence for a similar factor in the tuberculin p.p.d.-stimulated lymphocyte culture system. A cytotoxic factor, termed a lymphotoxin (135), which is responsible for the death of target cells by destruction of their plasma membranes, has also been described. Following phytohaemagglutinin activation of small lymphocytes the factor is apparently secreted independently

of DNA synthesis and before any evidence of morphologic transformation has occurred, and its production continues for long periods in culture.

The lymphocytes from some patients with chronic mucocutaneous candidiasis have been shown to respond *in vitro* to the candida antigen (14, 127). It has been further shown that following a transfusion of normal lymphocytes some of these patients acquired the ability to mount type IV delayed hypersensitivity reactions with erythema and induration (14). Thus there seems to be an immunological paradox in chronic mucocutaneous candidiasis, concomitant defective cutaneous cellular responsiveness but normal lymphocyte transformation. It has been postulated that the lesion in at least some of the patients with chronic mucocutaneous candidiasis, lies in their inability to produce lymphokines which would, in a normal person, lead to macrophage activation, erythema and induration. Another interesting feature in these patients is that endocrinopathy is sometimes an associated feature taking the form of hypoparathyroidism or hypothyroidism. Similarly, in a family described by Gatti *et al.* (32) two of three siblings had lymphopenic agammaglobulinaemia associated with a distinctive form of short limbed dwarfism and ectodermal dysplasia; and the authors suggested that a relationship may exist between osseous development and development of the immune system. One is thus encouraged to conjecture that in the endocrinopathy group, the clinical picture of candidiasis may be a manifestation of a thymic deficiency state, in that the ability to initiate synthesis and release of lymphokines might be controlled by the thymus as are other cellular immune functions. The other two endocrinopathies could then be linked together with the postulated thymic defect into one syndrome with a single underlying basic lesion, a generalized malfunctioning of all the branchial pouch derivatives. Support for this tentative suggestion can be derived from the work of Rocklin, Rosen and David (106), for they reported that lymphocytes from a patient with congenital thymic aplasia acquired the ability to produce macrophage inhibitory factor after a thymic transplant.

A dissociation has been reported by Matsaniotis, Tsenghi, Economou-Mavrou and Metaxotou-Stavridaki (78) between the skin test status and the *in vitro* response of the lymphocytes from a series of children challenged with Old Tuberculin. They found that following vaccination the appearance of an *in vitro* response always preceded the conversion of the skin reaction, and they also found that Old Tuberculin could induce transformation in lymphocyte cultures from children in whom the history and

skin reaction yielded no indication of previous sensitization. One interpretation that could be put on this data is that in children the delay between the onset of *in vitro* responsiveness and *in vivo* responsiveness may reflect the need for proliferation of the sensitized clone in the child's lymphoid tissues, to provide enough circulating cells so that in the event of a skin challenge sufficient lymphokine can be secreted to initiate a measurable skin response. Similarly the lack of skin response in those children who are positive *in vitro* may be physiological rather than pathological, in that they do not produce enough lymphokine in response to the test dose of Old Tuberculin, in contrast to the postulated total absence of lymphokine production in chronic candidiasis.

Production of interferon is yet another aspect of lymphocyte factor secretion of clinical portent. Green, Cooperband and Kibrick (39) found that human blood lymphocytes stimulated with tuberculin p.p.d., tetanus toxoid or diphtheria toxoid, produced an antiviral substance indistinguishable from interferon, and that the quantity of interferon produced was related to the concentration of antigen over a relatively narrow range (0.2 to 3 μg p.p.d. per ml of culture), although interestingly it was not related to the number of transformed cells. Maximal production occurred during days four to seven in their culture system. Richmond (103) similarly showed that in phytohaemagglutinin stimulated swine leucocyte cultures an inhibitor active against a range of viruses was produced which had several properties similar to those of interferon; and Wheelock and Edelman (133) reported that human small lymphocytes responded to 17D yellow fever virus infection *in vitro* by producing interferon.

It has been noted that patients suffering from various lymphocytic disorders are very susceptible to viral and bacterial pathogens and it is also known that suppression of cellular immunity unmasks latent virus infections. These considerations prompted Strander *et al.* (121) to investigate interferon production by human blood lymphocytes *in vitro* in some disease states, including Down's syndrome, blast cell leukaemia and chronic lymphatic leukaemia, where these cells are known not to be normal. Interestingly they found that the titre of virus induced interferon per ml of blood was very variable, but when equated to the cell content of the sample the interferon response per lymphocyte was fairly constant, except in chronic lymphatic leukaemia where there was a marked reduction in interferon production.

In two recent reports (22, 62) lists of lymphokines have been presented which seem to get longer and longer as the months pass. It is to be hoped that eventually there will be an agreed classification and nomenclature so that factors which are essentially

the same will go by the same generic title and those whose existence cannot be substantiated will disappear from the list, in much the same way as for example the original list of vitamins was rationalized. The production of lymphocyte factors *in vivo* extends and amplifies the response of small lymphocytes; this is a matter of necessity for owing to the extremely large range of antigen-specific recognition sites available or potentially available in the total lymphoid population the unit of stored information is small, and it is vitally necessary, if the body is to combat infection successfully, rapidly to initiate and augment a type IV reaction. Factors produced by a few sensitized small lymphocytes provide the means of local communication among mononuclear cells for they seem to act rather like local hormones. When they, and the part they play in effecting the repertoire of lymphocyte activity, are fully understood it will probably be possible to explain the physiology of delayed hypersensitivity reactions. What remains to be elucidated is the interplay between diffusible lymphokines and physical contact between mononuclears as to their respective roles in cell-to-cell communication, for it is known that lymphocytes and macrophages have the ability to participate in considerable gymnastics exemplified by rosette formation and emperipolesis which have hitherto been interpreted as forms of information transfer. Similarly a role in lymphocyte communication has been ascribed to the lymphocyte foot appendage (80). Possibly cell contact and lymphokine production are complementary processes.

From a clinical viewpoint a knowledge of lymphokine production would be of great value in understanding the impaired response, particularly in lesions like chronic mucocutaneous candidiasis. It may prove possible to confirm in the future that isolated defects in lymphokine production lead to specific clinical symptoms. Failure in interferon production, for example, may play an important part in permitting virus infection of patients with lymphocyte disease, although a relevant point was made by Strander *et al* (121), 'that the interferon response of the blood lymphocytes may not be so crucial in many viral infections as the effect exerted by the same cells at the portal of entry or in the regional lymph nodes'. On the other side of the coin, production of undesirable factors such as lymphotoxin, may be instrumental in killing transplanted organs. Detailed characterization of such factors and of their production pathway may lead to effective ways of inhibiting or blocking their effect.

PATHWAY

In much the same way that attention has come to be focused

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recently on one of the effector mechanisms, the production of lymphokines as a result of lymphocyte stimulation, so too has the afferent pathway of lymphocyte stimulation been subjected to greater scrutiny. In particular the binding of antigens to lymphocytes, the way the surface of the foreign antigenic determinant is recognized, and the way the resulting trigger impulse is conveyed to the lymphocyte nucleus are all important current research topics. Dwyer and Mackay (27), found that the early cellular response to immunization with flagellin included the appearance in human peripheral blood of blast-like cells which could bind antigen, and further that the increase in the total number of antigen-binding cells preceded the peak titre of circulating antibody by several days. The dynamics of this new cell population are worthy of much more investigation, as they have been noted after vaccination and during rejection crises (96). The presence of such metabolically active cells in the circulation is almost certainly the explanation for the elevated spontaneous transformation noted by Tennenbaum and his co-workers (122, 123), as an early indication of graft rejection both in human kidney recipients, and in canine cardiac allograft recipients. The same phenomenon almost certainly provides the explanation for the similar significantly increased spontaneous lymphocyte transformation detected by Parker and Lukes (97) during the 1968 Hong Kong influenza epidemic. It is distinctly possible that these activated cells may be some of the *in vivo* counterparts of the transformed cells seen *in vitro*. Such is their potential significance in a patient that they may become the object of routine clinical haematological reporting in the not too distant future. Reverting to Dwyer and Mackay's work with flagellin, the other notable change was that whereas prior to the injection only about 0.5 per cent of the lymphocytes reacted with ^{125}I-labelled flagellin, 14 days following immunization this increased to 4 per cent; in other words immunization led to an increase in the number of potentially reactive small lymphocytes in the peripheral blood. Data of this kind is providing information fundamental to the understanding of cellular immunological dynamics, and in the long term will probably prove of far greater significance to clinical immunology than will, for example, changes in a patient's antibody titres. Dwyer and Mackay were also able to show that antigen binding was blocked by antisera to both human IgG and light chains, but that cytophilic antibody was not responsible for the antigen capture phenomenon. Whether or not their work related exclusively to cells involved in Type II reactions or whether there was a Type IV component to the cell donor's immune status is not known, but certainly this autoradiographic approach

could be most valuable in delineating the antigen receptor or recognition site on lymphocytes. This leads naturally to a consideration of one of the most fascinating and central puzzles of contemporary immunology; namely, what is the nature of the antigen recognition site on small lymphocytes responsible for type IV reactions? There has been a spate of publications which have tended to implicate the light chains of immunoglobulins; Greaves, Torrigiani and Roitt (38) reported that rabbit anti-human light-chain antibodies used *in vitro* in amounts below the threshold which itself stimulates transformation, would cause the suppression of both the mixed lymphocyte reaction and tuberculin stimulation. The suggestion made by these workers was that anti-light-chain antibodies bind to the lymphocyte surface thereby blocking antigen binding either by steric hindrance or as a result of a configurational change. A similar line of thinking is to be found in the paper by Warner, Byrt and Ada (131), their findings leading them to propose that the recognition unit on CBA-mouse spleen or peritoneal cells was an immunoglobulin molecule.

In an attempt to explain some of the phenomenon known about lymphocyte stimulation *in vitro*, Coulson (19) postulated the existence of an antigen recognition site as a special immunological microstructure built into the lymphocyte membrane. Such a site would seem to have many features in common with the delayed hypersensitivity antigen recognition site postulated by Humphrey (50) and it is possible they are one and the same thing, which would be in keeping with the general view that *in vitro* stimulation via the recognition site is representative of *in vivo* type IV immunological responsiveness. A further point made in Coulson's hypothesis was that the structure of the surface recognition site rendered it antigenic when lymphocytes were injected to produce anti-lymphocyte serum. The important antibodies in anti-lymphocyte serum may be those whose specificity is directed against the recognition site, and that the combination of these particular antibodies with the site may be responsible for denying access to antigenic molecules. To explain how immunological combination at the lymphocyte surface could initiate nuclear activation, Coulson proposed the existence of a recognition pathway which was triggered off by microdeformation of the membrane at the recognition site and initiated a chain of biochemical events starting with cholinesterase or ATPase. Various by-pass and blocking mechanisms were also proposed, in particular it was suggested that phytohaemagglutinin stimulation resulted in a non-specific activation of the recognition pathway of all thymus-dependent lymphocytes by by-passing the recognition

site. Recent work by Revillard and Brochier (101) appears to fit the hypothesis, as lymphocytes from patients on a low dose of anti-lymphocyte serum (4–12 mg/kg/day) were found to be unresponsive to antigen *in vitro* although they were stimulated normally by phytohaemagglutinin. In addition to thalidomide and measles virus, amantadine (12) must be added to the list of agents that block antigen stimulation, but not phytohaemagglutinin stimulation, *in vitro*, conceivably by interfering with the same part of the pathway. Glasgow *et al.* (33) have isolated an alpha-globulin from human plasma that suppresses both phytohaemagglutinin and antigen induced proliferation of human lymphocytes *in vitro*, and which may be immunoregulatory *in vivo*. Possibly this factor blocks the pathway somewhere along the same segment where steroids and chloroquine are effective, i.e. after the phytohaemagglutinin by-pass.

The clinical significance of the postulated intracellular lymphocyte stimulation pathway, and the importance of a detailed picture of the recognition site lie in the fact that armed with this knowledge it may prove possible to engineer a more rational pharmacological attack on the small lymphocytes, incorporating specificity into immunosuppression. For one can contrast the input side of lymphocyte stimulation with its exquisite specificity, to the effector side of the activated lymphocyte. In the latter circumstance the lymphokines that mediate the repertoire of lymphocyte effects appear not to be antigen specific, so that therapeutic measures directed at this level would damp responses to both graft and pathogen alike. The same criticism can be levelled also against those agents that impede protein or nucleic acid synthesis; they are unable to discriminate between the different antigenic stimuli. For an immunosuppressive agent to be geared to a specific antigen it must operate at the level of the recognition site, or on the intracellular pathway soon after the initiation of stimulation. It is obvious that when the recognition site is more fully characterized and when the stimulation pathway is worked out we will be a lot nearer really selective immunosuppression; studies on these events are among the most exciting happenings in immunology at present.

GENERAL MEDICAL CONDITIONS

There is an ever-increasing number of disease states where the patient's lymphocytes have been found to have an impaired *in vitro* response to phytohaemagglutinin. Thus coincident with the prodromal phase of acute gastro-enteritis of unknown aetiology McIntyre and Cole (84) noted a remarkably low incorporation of ^{32}P into DNA in phytohaemagglutinin cultures; and Thomas,

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Clements and Naiman (125) noted a significantly depressed mitotic index during the acute phase of respiratory infections. Interestingly, plasma from these patients had no inhibitory effect on normal cells stimulated with phytohaemagglutinin. Bergman, Borgström and Tärnvik (9) found that 24 hours after cholecystectomy the response to phytohaemagglutinin was reduced in seven patients whether the cells were cultured in autologous or homologous plasma, and that the response began to recover within 10 days of the operation. Serum samples from the seven patients did not affect the phytohaemagglutinin response of normal lymphocytes.

On the other hand the impaired response to phytohaemagglutinin has been shown in a certain number of cases to be due to a serum factor, and in these cases one possibility to consider is that this may reflect only the general poor health of the patient and may have the same significance as a raised ESR. The same process that leads to quantitative and qualitative changes in the serum protein profile which are manifest as changes in the ESR may also lead to the non-specific production of proteins that inactivate or neutralize phytohaemagglutinin, or interfere with its transfer or binding site at the cell membrane. Knowles, Hughes, Caspary and Field (58) found, for example, that serum from patients with acute multiple sclerosis inhibited the spontaneous transformation of normal lymphocytes in culture as measured by tritiated thymidine uptake. Similarly, sera from patients with active multiple sclerosis were shown to inhibit phytohaemagglutinin transformation (112). In patients with secondary syphilis, Levene, Turk, Wright and Grimble (67) demonstrated the presence of a plasma factor which depressed the response of normal lymphocytes to phytohaemagglutinin. Similarly plasma from some patients with ataxia telangiectasia inhibited the response of normal control lymphocytes to phytohaemagglutinin (82); in addition there was suggestive evidence that elevated levels of α_2 globulin may have been responsible. Serum from patients with viral hepatitis when added to normal leucocyte cultures resulted in some inhibition of the phytohaemagglutinin response (134), and a serum factor in patients with regional ileitis has also been detected that depresses the response to phytohaemagglutinin (71). In the latter case it has been suggested that the serum factor serves to prevent excess proliferation of the lymphocytes and so helps to damp down the disease process. In other words the action of the depressive factor has been interpreted as an extension of an as yet unidentified physiological homeostatic mechanism. If this concept is valid then there is the exciting possibility that natural substances might be isolated which could be used specifically to control

lymphocyte proliferation *in vivo*. Certainly the results of McIntyre and Cole (84) dealing with the hour-by-hour variation in the response of normal lymphocytes to phytohaemagglutinin also seem to point in the same direction, and there may be some justification for their hypothesis that the normal immune system is subject to a form of 'regulation'. It remains to be seen of course if the inhibitory factor in all these disease states is the same thing or if it is due to a variety of different proteins, the latter eventuality would tend to militate against a specific homeostatic mechanism.

The lymphocyte response to phytohaemagglutinin *in vitro* has also been found to be reduced in a specially selected group of schizophrenic patients who had not had any medication for 6 months or more prior to the culture (75, 76). However, Malacarne and Dallapiccola (74) have presented evidence to show that on the contrary the lymphocytes of schizophrenics appear to have a greater inclination to spontaneous mitoses than normal. Patients with sarcoidosis have been found to have significantly reduced transformation rates which were most marked in those patients with progressive disease regardless of whether or not it affected the lymph nodes exclusively, or whether it had also affected the lungs. Stable sarcoidosis patients or those in remission, did not show this pronounced effect (61). Similarly in patients with severe uraemia Huber, Paster, Dittrich and Braunsteiner (49) reported an impaired response to phytohaemagglutinin obtaining only 22 per cent transformed cells compared to the 84 per cent in control cultures. With moderate uraemia (i.e. BUN less than 100 mg/100 ml) there was hardly any depression. Rössler, Havemann and Dölle (108) were able to distinguish patients with infectious hepatitis and chronic active hepatitis whose lymphocytes were unresponsive to phytohaemagglutinin, from patients with alcoholic or drug-induced hepatitis where there was a normal response. On the other hand Warnatz (130) described a normal phytohaemagglutinin response in his group of chronic hepatitis patients although it is true only two out of the twenty cases fulfilled the criteria of chronic active hepatitis. Willems, Melnick and Rawls (134) found that leucocytes from hepatitis patients were hyporesponsive when stimulated with phytohaemagglutinin; this effect was observed early in the illness and the authors attributed it to an alteration of the metabolic response of the circulating lymphocytes. This result would appear to support the findings of Rössler *et al.* (108). Brown, Taub, Present and Janowitz (13) reported that the uptake of iododeoxyuridine after 3 days of culture with phytohaemagglutinin and Concanavalin A showed that patients with regional ileitis had an abnormally low response when compared to a control group. All the patients had active

disease but were not receiving corticosteroids or other immunosuppressive therapy.

In vitro stimulation of lymphocytes from patients with the Guillain-Barré syndrome by an antigenic preparation of peripheral nerve led Knowles *et al.* (59) to postulate that in this condition there was a specific sensitivity on the part of circulating lymphocytes to a putative peripheral nerve antigen. In the seven patients investigated the average interval between the onset of the syndrome, and the lymphocyte testing was $1\frac{1}{2}$ years with a range of 1 month to 4 years. The lymphocytes from these patients failed to respond to basic protein of central origin (encephalitogenic factor), and lymphocyte cultures from patients with other forms of peripheral neuropathy and from controls showed no response to the nerve tissue. Bartfeld and Atoynatan (7) reported that human brain extract, encephalitogenic factor and cerebrospinal fluid produced significant transformation in leucocyte cultures from patients with multiple sclerosis, but not from patients with amyotrophic lateral sclerosis or control donors. However, there appeared to be no correlation of the degree of activity of the disease and percentage transformation. A similar result had previously been reported by Frick and Stickl (30) and they interpreted their results as suggesting a delayed type hypersensitivity reaction in these patients directed against encephalitogenic factor and possibly against immunoglobulin determinants.

In other conditions with a possible autoallergic component mediated by sensitized lymphocytes, Warnatz (130) accumulated enough evidence to permit the suggestion that *in vitro* lymphocyte transformation would be a suitable test for the diagnosis and control of such disease states. In particular he demonstrated that in chronic hepatitis and in cirrhosis, autologous liver extracts stimulated increased transformation compared to control cultures. Similarly in lymphocyte cultures from rheumatoid arthritis patients, there was a fourfold increase in the stimulatory effect obtained with autologous or homologous synovial extracts when compared to lymphocyte cultures from patients with infectious joint diseases or osteoarthritis. However, Rothenberger and Thiele (109) were unable to confirm this result; in their study of thirty-five patients with a high titre of rheumatoid factor they found no evidence of lymphocyte stimulation by synovial extract as measured by the production of transformed cells or by thymidine uptake. Altered cellular reactivity to streptococcal antigens or human glomerular basement membrane might, it has been suggested (136), be partially responsible for the continuing disease process in progressive glomerulonephritis. They were able to show that lymphocytes from fourteen glomerulonephritic patients

responded to particulate streptococcal antigens, whereas those from patients with unrelated renal disorders did not respond.

Patients with amyloidosis were investigated by Lehner, Cameron and Ward (64) and found to have an impaired *in vitro* response to Herpes simplex antigen, but not to purified protein derivative, phytohaemagglutinin or Candida as judged by ^{14}C-thymidine uptake. The authors, motivated by the possibility that a defect of cellular immunity could be a factor in the aetiology of the condition, suggested that there might be a selective impairment of cell mediated immunity. This is a similar concept to that formulated by Rodey and Good (107). They suggested, in the course of the discussion of their finding of a diminished phytohaemagglutinin response following the administration of amyloidogenic agents, that a deficiency of thymic cell function may be an underlying factor in the pathogenesis of amyloidosis. Interestingly, Lehner *et al.* (64) found that lymphocyte transformation was stimulated by a crude extract of amyloid fibrils in four out of seven patients with amyloidosis, and furthermore in three out of eight controls. The extract was prepared from the liver of a patient with primary amyloidosis by differential centrifugation. Further investigations of patients with amyloidosis along these lines, perhaps using a wider variety of amyloid preparations should yield valuable results.

It might have been thought that in pregnancy there would have been a depression of lymphocyte transformation as the mother virtually tolerates a homograft and there are many associated metabolic and hormonal changes, but Thiede, Choate and Dyre (124) reported that phytohaemagglutinin evoked the same response in lymphocytes from pregnant and non-pregnant females. A most interesting study of the lymphocytes of mothers in early pregnancy was made by Walknowska, Conte and Grumbach (128), where they found evidence for a leakage of foetal lymphocytes into the maternal circulation at least as early as the fourteenth week of gestation. The karyotype of some of the lymphocytes from twenty-one pregnant mothers showed euploid metaphase figures with five small acrocentric chromosomes which could be interpreted as 46 XY. Subsequently nineteen of the twenty-one gave birth to male babies. On this basis Walknowska *et al.* hope to be able to identify abnormalities of chromosome structure and number in the foetus as early as the first trimester. The findings of Debray-Sachs, Bach, Bach and Dormont (23) may be related to the cell leakage described by Walknowska *et al.*, for they found that mixed cultures between lymphocytes of mothers and their babies of less than 2 months produced significantly greater transformation than comparable mixed cultures between mothers and children in their families more than 10 years of age.

An apparent correlation between the teratogenic effect and the lymphocyte inhibiting effect of a series of agents including thalidomide, steroids, chloroquine, rubella virus and measles virus, was pointed out by Coulson, Summers and Inman (21), and they suggested that inhibition of lymphocyte transformation *in vitro* might provide a useful empirical screening test for teratogenic side effects of drugs. Schwartz, Stenzel and Rubin (117) also suggested that human lymphocyte culture had an important role in the evaluation of drugs. In particular, they found that a new immunosuppressant, cinaserin, which used at a concentration of 7×10^{-5}M inhibited phytohaemagglutinin stimulated protein and DNA synthesis by more than 60 per cent. It appeared to be effective early in the activation of the lymphocyte for its effect could be reduced by washing out the drug during the first 4 hours.

Lymphocyte cultures also provide an easily accessible source of somatic cells to investigate inherited enzyme disorders and other metabolic defects. Thus Lazarus, Vethamany and Volk (63) found lipid cytosomes in phytohaemagglutinin stimulated lymphocytes from patients with Niemann-Pick Disease, which were not present in similar cells from control subjects. This suggested that these structures were formed in the cells themselves and did not represent phagocytosed material, in addition it was claimed that the lipid cytosomes resembled those seen in rectal mucosa and in lymph nodes. Foley, Danes and Bearn (28) pointed out that short-term leucocyte cultures circumvented the technical problem of skin fibroblast cultures and provided a simple and more rapid screening technique to detect the heterozygous state for the mucopolysaccharidoses. They investigated seven individuals with either Hunter's, Hurler's or Sanfilippo's syndrome and also some of their parents; leucocyte cultures from the seven propositi and from eight heterozygous individuals showed marked cellular metachromasia. Cells examined on the third day of culture had fine metachromatic granules dispersed throughout the cytoplasm which by the seventh day were localized to the juxtanuclear area as a dense zone of pink staining material.

In a study by Diamond, Friedland, Halberstam and Kaplan (24) the leucocytes of gouty subjects and normals were investigated to establish the comparative rates of incorporation of glycine into nucleic acid purines. In the case of the fifteen adult donors with primary uricaemia all medication was stopped 75 hours before the leucocytes were separated from the blood. The authors ruled out dilution effects due to the different sizes of intracellular glycine pools, and were able to show that the mean incorporation rate of the labelled glycine into adenine and guanine was 1.8 times greater in the uricaemic patients than in the normals.

In a number of rarer medical conditions with associated immunological defects lymphocyte tissue culture has also been used in an attempt to analyse the immunological component. Thus Lux *et al.* (70) exploited tissue culture techniques in their extensive study of two children with cartilage-hair hypoplasia. Both patients had a grossly impaired response to phytohaemagglutinin which was not due to a plasma factor, and the lymphocytes of one of the children were shown to be unresponsive in mixed lymphocyte culture. This lymphocyte malfunctioning was thought to be the basis for their diminished cellular immunity and their nearly fatal varicella and recurrent respiratory tract infections, despite the fact they had normal or elevated salivary and serum immunoglobulin levels. McFarlin and Oppenheim (82) found reduced lymphocyte transformation rates in about two-thirds of patients with ataxia telangiectasia when they were stimulated with non-specific stimulants; in about half the patients a serum inhibitory factor accounted in part for the reduced responsiveness, but in 25 per cent of cases the defect appeared to lie in the cells themselves. Similarly patients with the Wiskott-Aldrich Syndrome exhibited diminished delayed hypersensitivity *in vivo* and defective lymphocyte transformation *in vitro* (95).

Following the observation that lymphocytes from patients with Down's syndrome appear to be more sensitive to dilute phytohaemagglutinin than normal lymphocytes, Matte, Sasaki and Obara (79) investigated a Down's patient with a chromosome mosaic. Blood and skin cultures revealed that the trisomic cells were responsible for only 5 to 6 per cent of the mitotic figures, but after 72 hours of culture the mosaic case produced a transformation response between that of the normal range and the enhanced response of pure trisomics. Sasaki and Obara (111) also found that mixed lymphocyte cultures between patients with Down's syndrome, or between Down's syndrome patients and normal subjects showed a strikingly increased response with a much higher mitotic activity than in mixed cultures between normal individuals. The greater proliferative potential of Down's lymphocytes may be caused by a genetic factor in these patients and it has been suggested that in may be related to the high incidence of leukaemia in this condition.

The investigation of a wider spectrum of disease states by the lymphocyte tissue culture technique that has taken place in recent years, has already yielded one important dividend and that is the finding of plasma factors which can inhibit lymphocyte transformation. The key point about the plasma factors is really whether they are *specific* substances, in which case they could be part of a physiological regulatory mechanism, and perhaps

lymphocyte tissue culture could be exploited to delineate the role played by such substances, particularly for example the α_2 globulins. The situation is, it is true, complicated by the fact that in some cases the decreased responsiveness is due to the cells themselves and this may reflect the ability of the lymphocytes to effect rapid population changes. Possibly, in some disease states or in the incubation period, the population of thymus-derived lymphocytes in the peripheral blood is rapidly depleted by their withdrawal into the lymphoid organs. However, it bears reiterating that if the depression of lymphocyte response proves to be more than an epiphenomenon arising from non-specific changes in the serum protein profile, then the cynical viewpoint that equates decreased responsiveness with an increased ESR may be justified.

To the practising clinician a culture of the patient's lymphocytes in an incubator is two things. Firstly, it is a suspension of somatic cells that has been obtained from the patient with relative ease and which can be kept alive and made to manifest its activities in tissue culture. The lymphocytes have the same inbuilt errors of chromosomal composition, and genetic and enzymatic defects as the other somatic cells, and the biochemical or histochemical abnormalities that result from these defects can readily be assessed. Secondly, they are immunologically competent and provide a convenient 'biopsy' of the patient's delayed-hypersensitivity system; thus it is possible to see if the lymphocytes are competent by challenging with foreign antigens or non-specific stimulants, and further it is possible to assess whether they have been pathologically sensitized by exposing them to autoallergens such as synovial extracts or encephalitogenic factor.

MALIGNANT CONDITIONS

In order to try and assess the prognosis for patients with different kinds of malignant tumours, Savel (113) attempted to determine the degree of the patient's delayed hypersensitivity to saline extracts of the tumour. In his paper he described how with the lymphocytes from seven out of fifty-six patients studied, there was a definite stimulatory effect obtained as measured by tritiated thymidine uptake when an extract of the patient's own tumour was added as an antigenic stimulus to his lymphocytes *in vitro*. In these seven patients the tumours had a relatively favourable prognosis including one malignant melanoma which appeared to be regressing spontaneously. Jehn, Nathanson, Schwartz and Skinner (51) have similarly reported that lymphocytes from seven patients with malignant melanoma responded to challenge *in vitro* with autologous tumour extracts. They

suggest that further purification of the antigenic material in malignant melanomas may, among other things, offer opportunities for 'therapeutic approaches to this disease'. In the same context Moore (89), and McKhann (85) have been pursuing the possibility of 'cancer lymphotherapy'. With facilities for culturing large volumes of lymphocytes at their disposal they have attempted *in vitro* sensitization of lymphocytes to cancer cells, followed by their infusion into the patient in an attempt to control the primary and its metastases. Similarly, Frenster and Rogoway (29) showed that re-infusion of autologous lymphocytes activated *in vitro* with phytohaemagglutinin resulted in 50 per cent size reduction of the pulmonary metastases of certain tumours, notably malignant melanoma, Ewing's sarcoma and testicular carcinoma. These workers aimed at re-infusing at least one activated lymphocyte for each neoplastic cell present in the patient.

Lung cancer patients were investigated by Ducos *et al.* (25) and their lymphocytes were found to undergo almost the same degree of spontaneous transformation as those of control patients; however, when challenged with dilutions of phytohaemagglutinin the lung cancer subjects showed a considerably reduced response. Contradictory results have been reported concerning the effect of serum from carcinoma patients on the phytohaemagglutinin response of lymphocytes *in vitro*. Gatti, Garrioch and Good (31) found a diminished phytohaemagglutinin response in a large series of adult patients with non-lymphoid malignancies who were not receiving chemotherapy or steroids, when compared with age-matched controls. Washing the lymphocytes three times in culture medium prior to phytohaemagglutinin stimulation increased the response over that of the unwashed cells, and fresh plasma from most of the cancer patients tested inhibited the response of normal lymphocytes. The authors did not commit themselves as to whether the depressed responses obtained reflected mainly hypofunctioning of the cancer patient's lymphocytes, or a plasma factor from the cancer patient, for it seemed to vary from case to case. However, Golob, Israsena, Quatrale and Becker (34) were fairly convinced that their results showed that no significant difference could be detected between the response of normal lymphocytes grown in cancer serum and control serum.

A comparison by Nelson (91) of the results of antigenic stimulation of lymphocytes from sixteen patients with early cancer and matched controls was found to be inconclusive. A battery of five antigens was used, including candida, mumps, tuberculin p.p.d., streptokinase-streptodornase and trichophytin, and a consistently lower mean response was obtained in the cancer group. A lower

total response to the antigens by the cancer patients in twelve out of sixteen pairs was also observed, but the disparity was only significant at the 5 per cent level. Nelson concluded that the differences in lymphocyte culture response possibly reflected the weighting effect due to the impairment evident in more advanced cases; interestingly, he also found no evidence for a plasma factor in cancer patients which could suppress the lymphocyte response to stimulation.

The decreased responsiveness of lymphocytes from patients with chronic lymphatic leukaemia to phytohaemagglutinin *in vitro* has been re-investigated by Bouroncle, Clausen and Aschenbrand (11). Their work suggested that an important factor in the *in vitro* situation may be the delayed response of the leukaemic lymphocytes, which is in keeping with the work of Havemann and Rubin (43) who claimed to have demonstrated a primary proliferative abnormality of chronic lymphatic leukaemic lymphocytes when they investigated this delayed response. Rubin, Havemann and Dameshek (110) found that lymphocytes from patients with chronic lymphatic leukaemia with modestly elevated lymphocyte counts and mild disease had only a slight reduction in their response to phytohaemagglutinin and only a relatively slight delay. A rough correlation was found between the patient's progressive lymphocytosis and decrease in circulating immunoglobulins, and the *in vitro* depression and delay of phytohaemagglutinin response. A potentially important point in the paper from Bouroncle *et al.* (11) was the finding that patients appeared to reach haematological and clinical remission months before the lymphocyte transformation returned to normal. This seems to be a recurring point in lymphocyte investigations at present, that the classical staining techniques in current use do not yield enough information about the lymphocyte so that any assessment of the patient's lymphocytes must test their functional capabilities. Rubin *et al.* (110) have also suggested that 'a definition of chronic lymphatic leukaemia could well include, not only the findings in the blood and bone marrow, but a statement of the reaction of the lymphocytes to phytohaemagglutinin and of the immunoglobulin characteristics'. In much the same way, Bouroncle *et al.* (11) suggested that chemotherapy should be prolonged beyond the time of remission as judged clinically and by classical haematological techniques, until the *in vitro* tests showed that the *in vivo* population had returned to normal.

The reduced *in vitro* responsiveness to phytohaemagglutinin on the part of lymphocytes from patients with chronic lymphatic leukaemia, lymphosarcoma and Hodgkin's Disease was confirmed by Scheurlen, Pappas and Ludwig (115). Whereas healthy controls

produced between 60 and 80 per cent transformed cells, under similar circumstances chronic lymphatic leukaemics produced less than 10 per cent and both lymphosarcoma patients and those with Hodgkin's Disease produced scattered values between zero and 60 per cent. Essentially similar results were also reported by Ambs, Chesebro and Lagerlöf (1), and by Hersh and Irvin (46). Pentycross (98) found that in Hodgkin's Disease there appeared to be a partial association with staging, with low transformation rates occurring in advanced stages of the disease, but it was not possible to see any clear association with the clinical state of the patients or their therapy. Havemann (42) attributed the initial low percentage transformation of the lymphocytes from Hodgkin's disease patients to a delay in response similar to that seen in chronic lymphatic leukaemia. He also found, as had Pentycross, that in patients with a more localized form of the disease (Stages I and II) the percentage of transformed cells was greater than that found in patients with more generalized Hodgkin's. Just as laboratory strains of bacterial pathogens are used to determine the effectiveness of new antibiotics, so Hirshaut, Weiss and Perry (47) have used leucocyte culture lines as models for a study of anti-leukaemic agents. This approach prompts the consideration that in future there may be a case for examining leukaemic lymphocytes isolated from patients and cultured *in vitro* to determine the best combination of drugs to use, but if this is done the results of Astaldi, Eridani, Ponti, Valentini and Giangrande (3), must be taken into consideration. They found, disconcertingly, that the effects of antimitotic drugs were not necessarily the same *in vitro* and *in vivo*; thus 5-fluorouracil showed a lower activity *in vivo* compared with its *in vitro* effect, while in the case of vinblastine the reverse was true. Hirshaut *et al.* (47) found that 6-mercaptopurine at doses approximating to one-tenth of those achieved clinically permitted the leucocytes to grow for several days before dying. Similarly methotrexate still permitted some growth for 48 hours, but vincristine seemed to be effective immediately. A point that Hirshaut *et al.* were at pains to stress was the importance of considering the drug concentration and the time of exposure in cancer chemotherapy, to obtain maximum destruction of leukaemic cells with minimal effects on normal cells. They suggested that high dose therapy should be attempted in leukaemia particularly to provide cytotoxic levels in more inaccessible areas.

The technique employed by Hirshaut *et al.* is similar to the one that Schwartz *et al.* (117) used to assay the possible immunosuppressive action of cinaserin and provides another example of the great potential that the lymphocyte tissue culture technique

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has for the screening and assaying of drugs. From the viewpoint of cancer therapy the other greatly exciting prospect of lymphocyte tissue culture lies in lymphotherapy, the sensitization of large numbers of lymphocytes to tumour cells *in vitro* and their reinfusion into the patient. In addition, tissue culture provides another source of data to measure the progress of the disease in leukaemic patients; the behaviour of the leukaemic cells *in vitro* could be considered along with blood count and morphology in assessing the patient.

The impaired response of patients' lymphocytes *in vitro* seems to be a fairly general phenomenon in cancer patients and as in other medical conditions, it appears to have a dual origin; in some cases it is attributable to serum factors and in others to the cells themselves. By and large the variety of tumours which stimulate the production of plasma factors appears to add weight to the viewpoint that such factors are non-specific, for it seems unlikely that a wide range of different kinds of malignancies could all trigger off a specific lymphocyte regulation mechanism. However, the possible alternative merits consideration, namely that the very reason why this association is seen is because the only 'successful' cancers are those that produce lymphocyte-inhibiting factors or that damage the lymphocytes directly, or alternatively that malignancies only develop in those unfortunate people who for some reason have hyporesponsive lymphocytes. In a sense this would mean attributing the origin of a wide variety of malignancies to a specific defect in the body's cellular immune surveillance system, namely the hyporesponsive lymphocytes. Certainly lymphocyte tissue culture is proving a valuable investigative tool in cancer immunology but a caveat needs stressing, namely that the patient's lymphocytes may have been functioning quite normally when the tumour started growing, but after a period the lymphoid population may have been subjected to subtle non-specific damage as part of systemic effects of the cancer on all the body's systems. This would make it dangerous to generalize on the basis of cultures done in patients with established cancer about the role of lymphocytes, or rather the lack of it, in permitting growth of the malignancy in the first place. This possible controversy about what came first, the lymphocyte defect or the cancer, could only be resolved by a prospective study.

CONCLUSION

The lymphocyte tissue culture field has undergone a phase of rapid expansion particularly in the last few years and in so doing it has found applications in many fields of medicine. The expansion has been both in depth—in experiments designed to elucidate the

mechanism of activation, and in breadth—in the investigation of patients with an ever-increasing variety of diseases. Both in acute infections such as gastro-enteritis and respiratory infections, and in more chronic states, exemplified by multiple sclerosis, secondary syphilis and regional ileitis, changes in the patient's small lymphocyte population or serum characteristics are reflected in a departure from the normal of his phytohaemagglutinin response. In the various experiments of nature found in congenital immunological deficiency states, defects in lymphocyte function are reflected in abnormalities in the *in vitro* response; in fact these have been so well established now that the response to phytohaemagglutinin could safely be used for instance, to diagnose Swiss-type hypogammaglobulinaemia from Bruton-type hypogammaglobulinaemia. Lymphocyte tissue culture is proving useful not only in diagnosis but also in making a prognosis, thus an *in vitro* responsiveness to antigenic preparations consisting of saline extracts of the patient's own tumour may indicate a favourable outlook whereas lack of responsiveness indicates the reverse; similarly in Hodgkin's disease the degree of impairment of the lymphocytes *in vitro* is partially associated with the staging.

Currently a lot of thought is being given to the possibility that some of the plasma factors which can be shown to depress lymphocyte activity *in vitro* may normally play a part in regulating the activities of the lymphoid population *in vivo*. There may be a homeostatic system *in vivo* mediated via humoral lymphocyte regulators, hormone-like substances specific to the lymphoid tissue, which may possibly be α-globulins or may be transported with these proteins. This line of thinking is especially relevant to oncologists since a number of reports have appeared describing a decreased lymphocyte responsiveness in patients with malignancies. If cellular immune surveillance does play a key role in the detection and destruction of malignant transformation in somatic cells *in vivo*, then it is easy to envisage a defect in such a system leading to an increased incidence of malignancy in a patient. In effect this would mean attributing a wide range of malignancies to an underlying inadequacy in the patient's lymphocyte 'strike force'. Another construction that could be put on the same data is that many malignancies cause subtle damage to a wide range of organ systems and the same kind of pathological process that leads, for example, to the development of the anaemia seen commonly in cancer patients, could be the underlying cause of the lymphocyte dysfunction. The controversy as to which came first, the malignancy or the immunological defect, could only be resolved by prospective studies. Should the concept of plasma regulating factors prove to have substance then it would indirectly lend support to the idea

of thymus control over lymphokine release, so that in those cases of chronic mucocutaneous candidiasis with associated branchial pouch endocrinopathies it may be valid to maintain the unifying concept of a dysfunctioning of all the branchial pouch derivatives. Derangements of parathyroid and thyroid manifest themselves as endocrinopathies, while derangement of the thymus is seen in an inability to release lymphokines, such as macrophage inhibitory factor and chemotactic factor, with consequent susceptibility to candidiasis.

A useful clinical concept in thinking of the immunological significance of lymphocyte transformation is to equate the response to antigenic stimulation *in vitro* with type IV delayed hypersensitivity *in vivo*; although, admittedly, there are experimental systems where this correlation appears not to hold strictly 'se non e vero, egli e stato un bel trovato'. Thus in an analysis of the patient's immune system, lymphocyte tissue culture provides a long-felt need for an *in vitro* test of type IV delayed hypersensitivity with the attendant inestimable advantage that a tissue culture bottle instead of the patient's skin, is used as a vehicle for the experiment. A similar valuable rule of thumb is to equate the response to phytohaemagglutinin with the size of the thymus-dependent lymphocyte population, so that any diminution in response which cannot be explained as due to serum factors reflects a decrease in the numbers of the thymus-dependent small lymphocyte population in the blood. Hence phytohaemagglutinin stimulation provides a good assay method for *in vivo* thymus function, it permits in effect, an assay of thymus reserve. In an analysis of some of the rarer diseases one is able to gauge the extent of the involvement of thymus-dependent small lymphocytes. In some more common diseases phytohaemagglutinin studies have indicated that the thymus-dependent small lymphocyte population in peripheral blood is subject to what appears to be a rapid depletion; this has prompted the suggestion that in some disease states, or during their incubation period, the population of thymus derived lymphocytes in the peripheral blood is capable of withdrawal into the lymphoid organs. Another interesting facet of immunologically-competent cellular traffic relates to the movement of what could be one of the *in vivo* counterparts of the transformed cell in the blood stream. Waves of blast-like cells which can bind antigen have been described in the peripheral blood following vaccination and other immunological challenges, and these cells are probably responsible for the increased spontaneous transformation seen in cultures from such donors. As yet there are no clues as to what regulates the movements or the final destinations of such cells.

Newly characterized regulatory substances produced by lymphocytes on the other hand, are constantly being added to the lengthy lists of lymphokines, which supports the plea for a rational classification of these factors. At the same time increasing knowledge about lymphokines has led to an understanding of one, formerly, puzzling feature of the type IV response, namely, why the majority of mononuclear cells at the site of reaction are not specifically sensitized to the antigen employed. The role of lymphokines as a system of local intercommunication between mononuclears should be seen in a broad biological context, as it may yield valuable information on cell to cell communication systems. From the survival viewpoint in the case of individual humans, the value of the lymphokine system is that it promotes a rapid augmentation of a type IV response, so that although recognition of foreign antigens is vested in a small unit it can be followed rapidly by a large cellular infiltration. What remains to be elucidated is the interplay between diffusible lymphokines and physical cell membrane-to-membrane contact as alternate methods of communication in immunologically stimulated mononuclear populations; are they possibly complementary processes? The role of lymphotoxin as against membrane apposition, is an equally fascinating puzzle in the analysis of those situations where the target cell for the patient's cytotoxic lymphocytes is itself a human cell. This occurs in three situations; the first is in autoallergic states where an analysis of the pathogenesis has been assisted by showing, for example, that in cirrhosis and chronic hepatitis autologous liver extract stimulates lymphocyte transformation, and that similarly in rheumatoid arthritis the lymphocytes are stimulated by synovial extract. The second series of examples can be found in patients rejecting organ grafts where the lymphocytes' target is, for example, a newly transplanted kidney; in this situation an increase in the rate of spontaneous transformation of the peripheral lymphocytes heralds the onset of the rejection episode. In these two situations a detailed characterization of lymphotoxins and of their production pathway, could be the first step in unearthing an effective way of inhibiting or blocking the cytotoxic effect. The third series of examples is provided by the cellular immune attack on malignant cells, which, unlike the previous two examples, is a most desirable state of affairs; so much so that various attempts at lymphotherapy have been made, including sensitization of the patient's lymphocytes with tumour antigen or phytohaemagglutinin *in vitro*, and reinfusing the activated cytotoxic cells.

There is a great need for new techniques in following the stimulation process in its early stages and in trying to understand

the way the nucleus is 'switched on'. For this reason it is a fairly safe prediction that more and more attention will be devoted to the small lymphocyte during the first few minutes and hours of culture after the addition of the stimulus and less to the proliferation of transformed cells after 4 or 5 days in culture. A particularly exciting aspect of the stimulation pathway relates to the antigen recognition site, is it an IgX molecule on the cell surface, or is it something other than an immunoglobulin, 'another surface mechanism' (35)? Details of the recognition site microstructure and the way the immunological stimulus is transduced to initiate a membrane to nucleus pathway can only be guessed at for the time being; in the same way it remains to be seen whether or not the antigenicity of the recognition site can be exploited to mount a more specific and destructive attack. But one thing is certain and that is that the more we know about the site and the pathway, the nearer we will be to engineering a more rational pharmacological attack on the small lymphocytes, and also to incorporating specificity into immunosuppression. One potential application for the postulated pathway has already been suggested, for it was noted that the agents that blocked lymphocyte stimulation were also teratogenic; lymphocyte culture could thus conceivably find a use in screening new drugs for possible teratogenic effects. This is one of the many pharmacological applications of lymphocyte tissue culture: inhibition of phytohaemagglutinin stimulation may also be employed to assay for immunosuppressive properties in pharmacological agents, and lymphocyte tissue culture cell lines have been used as models for the study of antileukaemic drugs. This latter technique has already shown that methotrexate and 6-mercaptopurine have a delayed effect in contrast to vincristine which is effective immediately *in vitro*. Although conflicting results have been reported, it is possible that culture of lymphocytes from a patient on immunosuppressives may indicate how effective is the therapy: thus a diminished response to phytohaemagglutinin could indicate satisfactory treatment while a normal phytohaemagglutinin response could indicate an insufficient dosage. Conflicting results have unfortunately also obscured the application of lymphocyte tissue culture to the assessment of a patient's immunological sensitivity to a pharmacological agent; it would appear that vagaries in methodology argue for caution in accepting what may subsequently prove to be over-enthusiastic reports.

Discussion up to this point has centred on those activities of the small lymphocyte which are the expression of a small section of the lymphocyte genome, that controlling its activities as a specialized immunocompetent cell, but the vast majority of genetic information in the small lymphocyte nucleus is common to all

somatic cells so that lymphocyte tissue culture can also be a living biopsy of somatic cell activities. As such the lymphocyte may manifest all the same inbuilt errors of chromosome composition, gene and enzyme defects, so that the biochemical or cytochemical stigmata that result from such defects can be visualized; such an approach has already been used in patients with mucopolysaccharidosis, gout, Down's syndrome and Niemann-Pick Disease. Consideration must be given to the culture of lymphocytes when these cells are the primary diseased cells in the body, particularly, for example, in chronic lymphatic leukaemia when *in vitro* studies provide an unobscured look at the cancer cell. It is noticeable that those clinicians who have employed such an approach have commented that patients with chronic lymphatic leukaemia *appeared* to reach haematologic and clinical remission months before their lymphocyte transformation test became normal, and they have urged that a test of the functional capabilities of a patient's lymphocytes *in vitro* should form part of the routine haematologic assessment during follow-up.

As the lymphocyte culture technique has become more widely used, its clinical value has become more and more widely appreciated. The very range of disease states investigated from schizophrenia and disseminated sclerosis to Down's syndrome and gout, for example, serve to illustrate its wide applicability. There can be few laboratory investigations which have in such a short space of time, become useful investigative techniques in such a variety of conditions.

SUMMARY

Lymphocyte tissue culture has found application in a great number and variety of medical conditions; this review is an analysis of the recent voluminous literature, and aims at giving the busy physician a bird's-eye view of the present situation and the way it is developing. Basically the tissue culture procedure provides the clinician with information of two different kinds; firstly he can use it as a living biopsy of the patient's cellular immunity and carry out tests with it analogous to those done to assess the patient's serological status. Thus if the patient's lymphocytes are abnormally active in unstimulated cultures this probably indicates the transit of a pulse of activated cells resulting from an intercurrent immunological challenge. The number of thymus-dependent small lymphocytes in a blood specimen can be measured by phytohaemagglutinin stimulation *in vitro*; similarly, the ability of the lymphocytes to mount a delayed hypersensitivity reaction in the body can be assessed by their response to the same antigen *in vitro*. This response may be to foreign antigens, auto-

logous tumour antigens or autoallergic tissue antigens. Immunological functions apart, small lymphocytes *in vitro* are also a sample of the somatic cell population and as such may manifest the stigmata that stem from inbuilt errors of chromosome composition, or gene or enzyme structure. From the pharmacological viewpoint they provide a readily accessible source of fresh human cells on which the anti-proliferative, immunosuppressive or teratogenic effects of drugs may be assessed, and by virtue of their immunologic function, possibly any hypersensitivity on the part of the cell donor.

Some of the ideas that have emerged from lymphocyte tissue culture studies include the possibility of a physiological *in vivo* lymphocyte regulatory mechanism, and the concept of rapid blood lymphocyte population changes in some disease states or during their prodromal period. Attention has also been focused on the lymphocytes' antigen recognition site, and on the intracellular stimulation pathway. Factors released by the stimulated lymphocytes, lymphokines, can be interpreted in a clinical light as local hormones essential to delayed hypersensitivity and survival, or in a broader context as constituents of a novel intercellular communication system possibly complementary to direct cell-to-cell membrane contact amongst the mononuclears.

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