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lymphocyte stimulation

LYMPHOCYTE STIMULATION

Completely revised edition

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1975

NORTH-HOLLAND PUBLISHING COMPANY – AMSTERDAM / OXFORD
AMERICAN ELSEVIER PUBLISHING COMPANY, INC. – NEW YORK

Library of Congress Catalog Card Number: 74-83274

North-Holland ISBN: 0 7204 4039 4
American Elsevier ISBN: 0 444 10701 0

PUBLISHERS:
NORTH-HOLLAND PUBLISHING COMPANY – AMSTERDAM
NORTH-HOLLAND PUBLISHING COMPANY, LTD. – OXFORD

SOLE DISTRIBUTORS FOR THE U.S.A. AND CANADA:
AMERICAN ELSEVIER PUBLISHING COMPANY, INC.
52 VANDERBILT AVENUE, NEW YORK, N.Y. 10017

PRINTED IN THE NETHERLANDS

Preface to second edition

The tremendous expansion of interest in the lymphocyte, in the seven years since the first edition was prepared, has been at once encouraging and bewildering. The rewriting has taken over three years and much more time and effort than we had anticipated. Ch. 1 remains as in the first edition but the rest of the book has been completely rewritten. It was originally intended to include a chapter on 'Lymphocyte activation in relation to disease' but this was finally omitted to avoid delay in publication. Although all the chapters have been revised during the last few months there are limits to the number of alterations which can be made and we are well aware of many remaining imperfections and omissions. References to the literature could not possibly be comprehensive and if many pioneers in the various fields feel that they have not been given proper recognition it is because we have concentrated on providing the reader with good leads into the literature by selecting recent important papers, particularly those which contain a good bibliography. The flood of publications, often in new and inaccessible journals, has unfortunately increased the difficulties of the researcher not attached to a big centre. It is one of the many undesirable side-effects of rapid progress in any scientific field.

We have tried to be objective without being aloof, airing our views but restraining our prejudices. This has not been easy because we have both found that we are most likely to differ from the majority opinion when we know most about the particular subject reviewed. We do not pretend that we are ourselves always in agreement and readers may like to know that chs. 12, 13 and 14 were written by J. K., ch. 6 jointly and the remainder by N. L.

We are grateful to Tony Nash, Helen Chapel, Terry Burn, John Lane, Geoffrey Holmes, Sue Menzies, David Catty, Anne Cooke, Theresa Ahern,

Valerie Lindsay, Alan Lehmann, Stanley Handmaker, Steve Vaughan-Smith, David Hardy, Stella Knight and Professor Philip Gell for help and advice. One of us (J. K.) would especially like to thank Herb Cooper for advice and guidance and many stimulating discussions. Freda O'Reilly and Diana King are thanked for typing the manuscript and June Atherton for drawing some of the figures.

April 1974

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Contents

CHAPTER 1

The transformation phenomenon

1.1. The survival and growth of leucocytes in vitro

When leucocytes from the peripheral blood, suspended in their own serum and a simple culture medium, are left at 37 °C for several days in an airtight container, some of the leucocytes degenerate but others remain viable and morphologically little changed. The cells which degenerate are the polymorphonuclear leucocytes (PMN). The eosinophils survive with little morphological change. The monocytes, while still recognisable from their nuclei, have increased in size and a cytoplasm, containing large vacuoles and frequently ingested debris, is bounded by an ill-defined cell membrane. They have matured into cells indistinguishable from tissue macrophages or histiocytes. The lymphocytes, which are now the most numerous of the viable cells, are also largely unchanged. No dividing cells are present unless the cultures are continued for a long period (say 7 days or more) and even then are few in number. If the whole procedure is now repeated, but with the addition of a small quantity of an extract of red kidney bean, *Phaseolus vulgaris*, to the culture fluid, marked changes occur in the morphology of some of the cultured cells. Large, active cells appear, with large nuclei and prominent nucleoli and, on the third or fourth day of culture, mitotic cells are to be seen. From this superficially simple phenomenon, first clearly described by Hungerford et al. in 1959 and Nowell (1960) stems most of the work described in this volume. The large, active cells are variously referred to in the literature as blast cells, transformed cells or stimulated cells. These terms will be used throughout the book. The use of the term 'transformed' cell may be misleading since it is used in a rather different sense by virologists; but it was used before the more accurate terms 'activated' or 'stimulated' were commonly adopted and shows no sign of disappearing from the

literature. Occasionally the term 'blastoid cell' is also used and the more general term 'large pyroninophilic cell', which is usually applied to all blast cells found in lymphoid tissue regardless of origin, also includes cells of this type.

1.1.1. The lymphocyte origin of the blast cells

The recognition that mitotic cells could be regularly obtained from peripheral blood created tremendous interest among workers in the field of human genetics. Further technical advances followed rapidly. Low concentrations of colchicine were added to accumulate mitoses arrested in metaphase and, after Moorhead et al. (1960) had devised a fixing and spreading technique for the display of chromosomes, cytological analysis became practicable and reliable. The method has been in general use throughout the world since 1960 and many important contributions in the field of human genetics have been made with it. The investigation of the nature of the cell which is mitotically activated by substances in extracts of *Phaseolus vulgaris* (phytohaemagglutinin, hereafter referred to as PHA) was not, at first, a matter which was pursued with any urgency and the result does not seem to have been awaited with great interest. The responsive cell was generally thought to be a monocyte or a large lymphocyte. Carstairs (1961, 1962), working at St. George's Hospital, London, was the first to show clearly that the small lymphocytes were the source of the dividing cells. At this time, he noted, two contradictory views on the nature of the small lymphocyte were held. The first view was: 'The small lymphocyte is a poor sort of cell, characterised by mostly negative attributes; small in size with especially little cytoplasm, unable to multiply, dying on the least provocation, surviving in vitro for only a few days, living in vivo for perhaps a few weeks'. The other view, expressed by Yoffey and Courtice (1956) was: '... the small lymphocyte is a specialised form of mesenchymal cell in a resting, relatively inactive state and reduced to the smallest possible size for the purpose of easy mobilisation and transport through the blood stream'. The demonstration by Carstairs, that the small lymphocyte could be activated to a dividing state was thought to be evidence in favour of a pluripotential role for it, as may be judged from the title of one of his papers.

Carstairs treated blood with iron-carbonyl particles to remove phagocytes and with PHA to sediment erythrocytes and provide stimulant. The initial nucleated cell content of his cultures was 0.56×10^6/ml, of which 83% were small lymphocytes, 12% medium and large lymphocytes and less than 0.1%

monocytes. Within 24 hr of setting up the cultures there were many cells which resembled medium lymphocytes. At 36 hr many cells contained deeply basophilic cytoplasm and the nuclei possessed prominent nucleoli. At 48 hr mitotic figures were common and many cells which superficially resembled reticulum cells, plasmablasts or proerythroblasts from a megaloblastic marrow were observed to be present. Nucleoli were prominent and sometimes irregular in outline. In the cytoplasm a few cells had a sprinkling of granules similar to the non-specific granules of promyelocytes. Vacuoles were common in the cytoplasm. By the end of the third day these cells formed almost the entire population. During the time taken for the small lymphocytes to drop from 83 to 25% of the nucleated cells (about 36 hr) there was a decrease in total count of approximately 10%. After the first 38 hr the population of small lymphocytes decreased still further, but in cultures continued beyond the period of maximum mitotic activity (68 to 86 hr) the number of small lymphocytes began to increase. These morphological observations fitted well with the observations of Cooper et al. (1961) on the uptake of tritiated thymidine (^{3}H-TdR) into desoxyribonucleic acid (DNA) in PHA-stimulated leucocyte cultures. At 24 hr they found a sharp increase in ^{3}H-TdR-positive cells and at 70 hr 50% of the mononuclear cells were labelled.

1.1.2. Early observations of blast cells in leucocyte cultures

It might be presumed, from the accounts given in the preceding sections, and in the other chapters, that lymphocytes had not been cultured and observed to undergo morphological changes until the late 1950s. This presumption would be erroneous. The movements of rabbit blood lymphocytes kept at 37 °C were examined microscopically by Ranvier in 1875 and, as early as 1888 Renant was suggesting that lymphocytes circulated in vivo and that some transformed and differentiated into connective tissue cells (both authors quoted by Coulson 1966). To a certain extent what an investigator finds is influenced by what he is looking for. During periods when controversy concerned the relative importance of cellular and humoral immunity many experiments were designed to discover whether or not leucocytes (usually without attempt to subdivide them) produced active protective substances. It was established in the 1890s that bacterial agglutinins found in the blood of a patient were preformed and that the titre was unaffected by the removal of the leucocytes, although it was considered by Metchnikoff that some bactericidal substances in serum might arise from the destruction or injury of

phagocytes. When the opposing 'end-cell' and 'multipotent cell' theories became the principle subject of argument, the emphasis, in leucocyte culture experiments, shifted to morphological observations of the relationships between lymphocytes and other cells. Maximow (1902) found blast cells (he called them polyblasts) in inflammatory exudates. He suggested that some of these blast cells arose from the mobilisation of local fixed histiocytes, others from lymphocytes and monocytes. Maximow strongly supported the contention that blood lymphocytes transform morphologically and functionally under an appropriate stimulus. He later observed that if tissue extracts were added to plasma clot cultures of rabbit lymph node cells, the lymphocytes hypertrophied into blast cells.

Scientific advances are sometimes divided into those which would not have been possible without the advent of a new technique (e.g. cellular ultrastructure could not have been examined before the development of the electron microscope) and those which have not required any new technique or equipment, or even knowledge, and could have been made much earlier. The phenomenon of lymphocyte transformation (consider, in particular, the mixed lymphocyte reaction, ch. 7) must be considered to be in the second category. Discoveries not married closely to technological advance may meet the charge that they are not new discoveries at all, but have already been reported in papers which have escaped attention. Although often factually correct these charges may be misleading. It could truthfully be claimed that Maximow and others observed lymphocyte transformation during the early part of this century. But it would have been difficult to have distilled this simple fact from the early literature, or even with confidence, from Maximow's own papers. The blame for this, if it is not to rest with the original investigator, must rest with his contemporaries rather than with a later generation. Much of the early confusion arose from the complexity of the media used and the arbitrary inclusion of sera and tissue extracts of diverse origin. More arose from the conviction that macrophages and many other cell types could be derived from blood lymphocytes and the ease with which this conclusion was accepted. Some idea of the confused interpretations often placed upon experimental research which, by modern standards, was exceptionally painstaking and detailed may be obtained by reading the review by Bloom (1938): '... In normal blood in the first thirty-six hours great numbers of amoeboid cells were found which became more or less rounded up and resting at forty eight hours. Scattered among the numerous lymphocytes on the second day of culture many hypertrophic cells of mono-

cytic type were found which were arranged in groups and showed no mitoses. The cytoplasm of these cells seemed homogeneous and slightly basophile, the nucleus filled with coarse chromatin particles. In the protoplasm of these cells there were a few deep-blue nuclei of leucocytes. On the third and fourth days the number of lymphocytes continuously decreased and only occasionally is a normal lymphocyte found. On the fifth and sixth days the picture is dominated by cells with a basophile protoplasm and an eccentric, slightly indented nucleus which are (considered to be) hypertrophic monocytes. From these there are transitions to large, rounded cells, with darker, foamy basophile cytoplasm. They develop processes and some of them merge into fibroblast-like cells which have a weak basophilia and occasionally a slightly eosinophile cytoplasm ...' The following could be interpreted to be an early example of antigen-specific transformation: 'When rabbit lymphocytes were injected into living connective tissue and the connective tissue explanted with embryonic extract, the lymphocytes turned very rapidly into macrophages while they remained unchanged for many days if bone-marrow extract was used. The addition of whole *Ascaris* extract (1 in 20 in Tyrode solution) did not affect the lymphocytes. But when the lymph of *Ascaris*-immunised rabbits was injected into rabbit connective tissue with bone-marrow extract and *Ascaris* extract and cultured, myelocytes developed in 6 days in 3 out of 5 sets of cultures from the explanted small and large lymphocytes ...' Maximow deserves the credit for insisting that lymphocyte transformation was possible. He observed: '... Occasionally some of the lymphocytes in chick leucocyte cultures develop large amounts of deeply basophilic cytoplasm, the nucleus becomes vesicular and the nucleolus very large. Such cells are obviously undergoing a transformation into haemocytoblasts. In the course of the first ten to fifteen hours in vitro the lymphocytes and monocytes hypertrophy into small macrophages, usually with very few mitoses accompanying this process ...' Chrustschoff (1935) also impresses one as being ahead of his time in developing a leucocyte culture technique (using a medium containing embryo extract) suitable for the study of the karotype of dividing cells. Timofejewsky and Benewolenskaja (1926, 1928) appear to have been the only other authors who drew attention to the fact that mitotic activity might sometimes be found in leucocyte cultures and they almost certainly observed a leucocyte activation induced by tubercle antigens. When optimal concentrations of virulent mycobacteria tuberculosis were added to leucocyte cultures, lymphocytes and monocytes, they found, transformed into polyblasts and, eventually, into epithelioid cells. The selection, for com-

mendation, of those authors who obtained positive results, does not reflect discredit on those who, probably through failing to introduce foreign proteins in sufficient quantity into their media, obtained negative results and truthfully reported them. Veratti (1928) considered that lymphocytes did not undergo change in culture and that the macrophage-like cells which appeared were derived from monocytes. Lady Jean Medawar (1940) also found no evidence of transformation of lymphocytes into macrophages in cultures but was able to correlate the number of mononuclear phagocytes which appeared with the number of monocytes present in the original cell population.

1.1.3. PHA-induced transformation

The survival and reactivity of cells in vitro is liable to be affected by alteration of any of a large number of environmental factors such as pH, O_2 and CO_2 tensions, temperature, the nature and shape of the culture vessel, serum concentration, cell concentration, the culture medium and whether the cells are allowed to settle at the base of the container, or are mixed by continuous agitation. Conditions which are thought to be optimal are outlined in ch. 3. Lymphocytes may be spun down from serum or plasma and resuspended in fresh serum plus medium without damaging the cells, provided that mild centrifugation conditions are used. Repeated washing of peripheral leucocytes has been shown by Caron (1967) to cause an inconstant reduction in their ability to respond to PHA. The effectiveness of PHA in cultures is dependent, principally, on using a suitable quantity of an active preparation. The importance of the 'dose variable' was realised by Newsome (1963). In cultures containing 1% PHA, 4–13% of the lymphocytes underwent morphological charge compared with 36–76% using 10% PHA. Dose-response curves for one of the varieties of PHA in common use, PHA-P (see ch. 11 for definition), were prepared by MacKinney (1964). A maximal response was obtained with 8.5 μg PHA-P/ml of culture fluid and a 50% response with 3.0 μg/ml. Approximately constant mitotic activity was obtained in the range 8.5 to 100 μg/ml. Very high concentrations of PHA-P were found by Wilson (1966) to be inhibitory and the same is true of PHA-M. PHA may be regarded as a universal stimulant for lymphocytes. It will stimulate a high proportion of the lymphocytes of most individuals and it will stimulate the lymphocytes of many animal species. Unresponsiveness of a lymphocyte population, when it is encountered, may be due to an abnormality of the cell population of that individual or to inhibitory substances in the serum.