Leukocyte Antigens and Antibodies

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By

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Preface

The field of leukocyte immunology has shown rapid expansion in the last few years. The present book intends to amalgamate and to a considerable degree assess certain results, theories and hypotheses published to date. It may have some additional value in bringing before the American reader the results of studies which in great part have appeared in foreign periodicals. Since no previous work of this kind has appeared in English, I happily do not have to justify the publication of "another" text in a worn field. The book will, I hope, be useful for two reasons. First, it may allow the interested student or clinician to approach some important aspects of leukocyte immunology without the immediate necessity of wading through a large but relatively unassembled literature. Second, a careful and detailed weighing of current evidence may greatly clarify and focus attention on certain major problems of leukocyte immunology as yet incompletely solved.

It seems pertinent to state precisely the scope of the book, what it is about and what it is not about. I have included only those broad aspects of the field with which I have had some personal concern either in research or in the practice of clinical pathology. The book, therefore, is chiefly concerned with humoral or circulating antibodies directed against leukocyte antigens, and with the theoretic ramifications thereof. What this includes may be seen by a glance at the table of contents. Two large aspects of leukocyte immunology are not included here. These are (1) the role of the leukocyte in the passive transfer of delayed hypersensitivity and (2) the possible role of the leukocyte in antibody transport or synthesis.

I wish to express my appreciation to Miss Patricia Doyle for technical assistance in my own studies, and to the Departments of Medicine, Surgery and Pediatrics, University of California School of Medicine, Los Angeles, for access to clinical material. These studies have been supported by grants from the Blood Bank of San Bernardino-Riverside Counties and the United States Public Health Service. I am also indebted to the staff of the Medical School Library for help in tracking down certain periodicals.

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Introduction

In the first half of the twentieth century a moderate but rather sporadic amount of investigation was done concerning heterologous immune sera directed against leukocytes. By "heterologous" I refer to systems in which an individual of one species is rendered immune to tissues of another species. This contrasts to the "homologous" situation, in which both individuals are of the same species (although not of the same inbred strain). In 1952 Dausset and Nenna [121] described a homologous antileukocyte antibody in the serum of a patient with agranulocytosis. In the same year Moeschlin and Wagner [377] reported the association of leuko-agglutination with pyramidon sensitization in the human. Since these two reports, antigen-antibody reactions of white blood cells have increasingly assumed the status of a major line of research activity, particularly in immunohematology. Leukocyte iso-antibodies subsequent to transfusion, the relation of leukocyte auto-antibodies to certain neutropenic states, the immune nature of the L. E. phenomenon, the occurrence of leukocyte antibodies in some instances of skin and tumor homografting—these areas in particular have been the subject of much investigation.

Several considerations lead me to believe that the subject may have an even more rewarding future. While a considerable body of knowledge has been accumulated about protein and other inert antigens, about erythrocytes and bacteria, we know comparatively little about humoral immune systems involving living nucleated mammalian cells, for example, the leukocyte, the epithelial cell and the fibroblast, to name only a few. Our lack of knowledge regarding such cells from the standpoint of heterologous immunity is large; from that of homologous immunity it is fairly staggering. Progress in elucidation of mechanisms particularly of auto-immune disease phenomena, and of the homograft reaction, requires that this ignorance be amended. To what extent, it may be asked, is our general knowledge of the immunology of nonliving material or of simple cells applicable to the homologous immunity of complicated cell systems? To what extent must entirely new mechanisms and concepts be brought forth? The leukocyte is rather well adapted to inquiries of this sort. It is

somewhat easier to obtain in pure form than most other body cells. Knowledge of its physiologic and biochemical properties is reasonably broad, and these properties in most instances are not too difficult to measure. Aberrations under the impact of an immune reaction are therefore amenable to investigation.

Contents

CHAPTER ONE

Technics for the Study of Leukocyte Antigens and Antibodies

MATTERS OF TECHNIC are usually relegated to an appendix. In the present subject, however, the main problems still hinge very largely on technical considerations. While a detailing of technical minutiae makes for a rather dry beginning, it can hardly be helped. A critical appraisal of the various methods for studying leukocyte immunology, with some comment on their shortcomings and their significance, seems a necessary background for the more theoretic presentation to be given in later chapters. The present chapter therefore will be concerned first with methods for isolating the antigen, i.e., the leukocyte or its parts, from humans and from laboratory animals, and second with methods for demonstrating reactions of these antigens with appropriate antisera.

Separation of Leukocytes from Human Blood

In separating leukocytes from whole human blood for immunologic study one must first decide whether to use blood rendered incoagulable by defibrination, decalcifying resins, or anticoagulants. If anticoagulants are employed, which are best for the purposes at hand? Also, should glass or nonwettable (plastic or siliconized) containers by employed? Some information is available on most of these points. Defibrination has the advantage of getting rid of about 90 per cent of the platelets, which become stuck to the clot. There are, however, several disadvantages. In normal blood at least 20 to 30 per cent of the leukocytes are also lost in the clot.[543, 545] Furthermore, defibrination provokes a fair degree of nonspecific leukocyte clumping. The degree of cell loss is kept minimal if a minimum number of glass beads is used in the defibrination. Three beads per 10 ml. of blood are adequate. The swirling of the blood should not be unduly prolonged as this too will markedly reduce the number of harvested leukocytes. Percentage of the total yield is least satisfactory with large volumes of blood, which require a comparatively longer time for complete defibrination. The leukocyte morphology of recently de-

fibrinated blood is relatively normal, and the cells show active ameboid motility. The leukocytes from defibrinated blood have been used by many workers in performing leuko-agglutinin tests,[104, 197, 260, 405, 426] and no adverse reports are on record. Exchange resins such as Dowex 50 may be employed to obtain uncoagulated blood. Methods for using resins in this manner are given by Stefanini and Dameshek,[489] and in greater detail by Dausset et al.[119] Besides decalcification, the resins effect partial removal of platelets. Leukocytes obtained from resin-treated blood have been employed in immunologic studies.[526] Such leukocytes seem well preserved biochemically as judged by lysozyme determinations [162] and by measurement of their respiratory and glycolytic activities.[320] The effects of anticoagulants per se on leukocyte immune reactions in vitro, particularly the leuko-agglutinin test, seem quite variable. Many divergent reports can be cited, even by the same authors. Dausset and Nenna [123] stated that oxalate, heparin and sequestrene may give false positive leuko-agglutinin reactions and that citrate is to some extent inhibitory to the leuko-agglutinin reaction. Elsewhere Dausset et al.[124] observed that heparin may give false positive leuko-agglutination, whereas oxalate, citrate and sequestrene are inhibitory. In other investigations Dausset [108, 125] employed citrated blood in performing leuko-agglutinin tests. Tullis [526] found that citrated plasma caused false positive results in immune studies involving lysis, agglutination and altered ameboid motility of leukocytes. However, Andre et al.,[18] employing citrate as anticoagulant in an extensive study of leuko-agglutination, noted 25 positive cases among 150 polytransfused patients, and entirely negative reactions among 200 normals. Citrate was employed by Milgrom, et al.,[354] in isolating leukocytes to be used in complement-fixation studies, and by Bagdasarov et al.,[22] in a large series of leuko-agglutinin tests. Under certain conditions citrate may inhibit "leukergy" of leukocytes and might therefore have additional value as inhibiting one of the nonantigen-antibody causes of leukocyte agglutination.[164] Flanagan and Lionetti [162] and Lochte et al.[296] presented biochemical evidence that ACD solution is a poor leukocyte preservative; however, leukocytes separated from whole blood via a citrate-fibrinogen solution showed active ameboid motility and vital staining for at least four hours.[21] Heparin has been successfully used by some workers in doing leuko-agglutination tests,[375] but Wasastjerna [545] noted that both heparin and oxalate may cause

nonspecifie leukocyte clumping. The concentration of heparin seems to be important, and too strong a solution may cause clumping.[163] Bessis [41] stated that some but not all samples of heparin will cause nonspecific clumping. Heparin does nevertheless appear to be a good leukocyte preservative when used in the proper concentration, as judged by ameboid motility [278, 543] and biochemical tests.[309] Brittingham [67] successfully used both sequestrene and defibrination in his studies and stated that all other anticoagulants tested were unsatisfactory. Sequestrene was also found suitable by Wasastjerna [544] and by Van Loghem et al.[302] On the other hand Killman [254, 260] found sequestrene, citrate and heparin to inhibit the leuko-agglutinin reaction. The best comparative study of the effect of various anticoagulants in the leuko-agglutinin test is in fact that of Killmann,[260] who preferred defibrinated blood. Finch and Detre [159] noted that sequestrene depressed the phagocytic activity of leukocytes for starch granules. Oxalate has been used for demonstrating heterologous antigen-antibody reactions of leukocytes [455, 498] but is probably not suitable in homologous situations.

The use of nonwettable versus wettable surfaces in studying leukocyte immunology can be briefly discussed. Dausset [124] found that siliconization of glassware may inhibit the leuko-agglutinin reaction. This has also been our experience. However, many investigators have successfully employed siliconized glassware in agglutination studies.[17, 197, 375] No experience has been reported with plastic containers.

From the above hodgepodge of conflicting observations and from our own experience the following suggestions may be offered. Either defibrination or exchange resins, especially the former, are recommended for tests in which an excess of platelets might cause interference. Defibrination, citrate or sequestrene are all probably suitable in setting up leuko-agglutinin tests. The last may be inhibitory to weakly reactive antisera but has the advantage over defibrination of yielding suspensions almost completely free of nonspecific clumping. Heparinized blood plus siliconized glassware is indicated for studies of leukocyte ameboid motility. Siliconization of glassware seems optional in other instances.

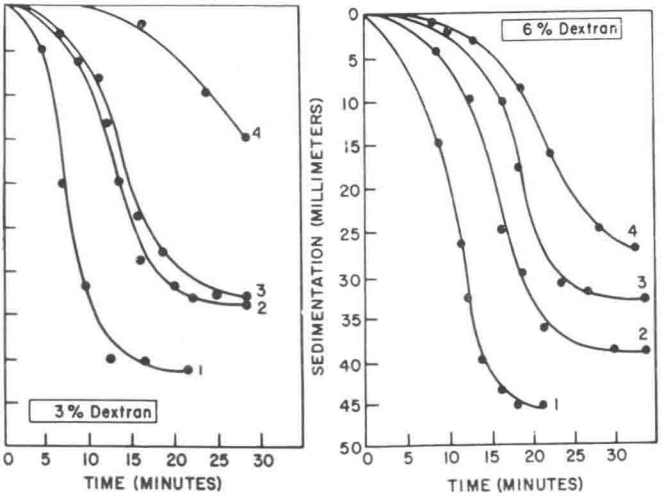
The next problem is that of separating or concentrating intact leukocytes from the uncoagulated whole blood. The remarkable plethora of methods published to this end indicate that no one

method is entirely satisfactory. The methods fall naturally into five categories, which are (1) sedimentation, (2) selective destruction of red blood cells, (3) the use of liquids of different specific gravities to separate the heavier erythrocytes from the lighter leukocytes, (4) centrifugation, particularly when combined with specialized apparatus and (5) a number of miscellaneous methods. Under the last, one may for convenience also include the isolation of human leukocytes from other sources than whole blood. We shall consider these categories in order. Aeberhardt's review of methodology may also be consulted,[2] and that by McKane and Ingram.[319]

Sedimentation

Before the value of specific sedimentation agents was appreciated, early workers obtained concentrated leukocyte suspensions by allowing leukemic blood to settle and drawing off the supernatant leukocyte-rich plasma. Erythrocytes settle more rapidly than white blood cells. This differential settling or sedimentation is greatly augmented by the formation of erythrocyte rouleaux. True agglutination of the red cells, such as caused by phytohemagglutinin [292] has a similar effect. Minor and Burnett [357] in 1948 were the first to employ a sedimenting agent (fibrinogen) specifically to separate leukocytes from whole human blood. Buckley et al.[70] nicely defined the variables that must be considered to achieve optimal separation with fibrinogen. More recently the plasma volume expanders have enjoyed much popularity. These include dextran [72, 197, 278] and polyvinylpyrrolidone,[63, 124, 244, 375, 405, 429, 430] as well as dextrin, glutamyl peptide,[523] gum arabic,[532] and other agents.[528] Oxypolygelatin has not as yet been employed in the isolation of leukocytes but might prove rewarding. It causes a marked increase in the sedimentation rate of red blood cells washed free of plasma.[149] The only comparative study on the use of several of the various agents is that of Skoog and Beck.[478] They compared the fibrinogen, dextran and phytohemagglutinin methods of isolating leukocytes. Employing 3 per cent dextran (molecular weight, 228,000) or 6 per cent fibrinogen, they considered that a dextran:blood ratio of 2 to 1 by volume, or a fibrinogen:blood ratio of 1 to 1 by volume, gave optimal results with regard to high leukocyte yield and low erythrocyte contamination. But their data require careful examination in the light of what one proposes to do with the leukocyte suspension. They were mainly concerned with harvesting

leukocytes for enzymatic studies. To obtain a suspension of nonclumped leukocytes from normal blood in as high a concentration per cubic millimeter as possible, and without centrifugation (which causes some nonspecific aggregation and deterioration [70, 309]) somewhat different ratios of sedimenting agent to blood might be optimal. FIGURE 1 illustrates this point, and a dextran:blood ratio of 1:4 may be seen to give an excellent suspension of antigen for agglutination tests.



CURVE	D:B	WBC YIELD	RBC IN SUPERNATE
		%	(PER CUMM)
1	2:1	76	59,000
2	1:1	40	14,000
3	1:2	78	25,000
4	1:4	72	12,000

CURVE	D:B	WBC YIELD	RBC IN SUPERNATE
		%	(PER CUMM.)
1	2:1	71	66,000
2	1:1	37	11,000
3	1:2	56	35,000
4	1:4	60	10,000

Fig. 1—Dextran-blood sedimentation curves obtained using dextran of molecular weight 228,000 (adapted from Skoog and Beck [478]).

Similar remarks apply to fibrinogen. Phytohemagglutinin, which has been used by Rigas et al.,[425] was less adequate as sedimenting agent than either dextran or fibrinogen, according to Skoog and Beck [478]; however, they as well as Valentine [530] obtained excellent yields employing a combination of fibrinogen and phytohemagglu-

tinin. Dextran and phytohemagglutinin can also be used in combination.[87] No comparative study involving polyvinylpyrrolidine is available. Dausset et al.[124] mentioned that dextran of molecular weight 100,000 gave somewhat more rapid sedimentation than PVP. Using 3.5 per cent PVP, Wasastjerna [545] reported an 80 per cent yield of leukocytes—a figure that is comparable to those with dextran or fibrinogen. Certain lots of dextran are injurious to leukocytes, as determined biochemically.[309] Tullis [526] has made similar comments about dextran based on ameboid motility studies. These injurious effects of certain lots are probably due to the presence of exceedingly high molecular weight polymers, the weight given by the manufacturer being only an average. After using dextran, fibrinogen and PVP rather extensively, I personally prefer the last.*

The following technic has proved useful. Three to 4 ml. of a 3.5 per cent solution of PVP or a 6 per cent solution of dextran is added to 10 ml. of whole noncoagulated blood. After thorough mixing by inversion the blood is pipetted into a second tube (this one siliconized), with care not to transport any foam into the second tube nor to get any blood on the tube wall above the fluid level. The size of the tube is fairly important. About a 15 × 1.8 cm. tube, inclined at a 45 to 60 degree angle, gives the best results for 10 to 20 ml. of blood. After 15 to 45 minutes of settling at room temperature, by which time the erythrocyte-supernatant juncture is visible as a sharp line, the supernatant is removed to a fresh tube. The blood can also be allowed to settle in an inverted siliconized syringe to which is attached a short length of plastic tubing, and the supernatant conveniently removed by pressing slowly upward on the barrel. This technic in a more complicated form was described by Klein et al.[264] If one is doing leukoagglutinin tests with normal blood cells, it may be desired to concentrate the final suspension by centrifugation. This should be done as gently as possible. Centrifugation for five minutes at 120 g with discarding of the upper one-third of supernatant generally suffices to bring the concentration to within 10,000 to 15,000 leukocytes per cubic millimeter. If one is harvesting leukocyte cream for tests not hindered by the nonspecific clumping of rapid centrifugation, an initial PVP: blood ratio of 1:1 is best.

Further separation of the leukocytes from erythrocytes in all the above cases can be obtained by repeated washing and sedimentation or centrifugation of the white cell suspension in a saline medium, according to technics of Maupin [314, 315] Robineaux et al.[429, 430] or Dausset et al.[118]; this maneuver takes advantage of the so-called "paradoxical sedimentation." I might say that I have found paradoxical sedimentation a very useful and efficacious procedure.

* A suitable polyvinylpyrrolidine is "Plasdone C," Antara Chemicals, New York 14. As for dextran, the Holland variety is, like their gin, the best available. Ask for "Poviet" (molecular weight, 160,000). Amstel brouwery, Stadhuuders Kade, Amsterdam.

Better yields and purer suspensions of leukocytes can of course be obtained by utilizing the blood of acute infection or leukemia having an elevated count. Such blood may, however, display excessive nonspecific clumping. This phenomenon has been labelled "leukergy" by Fleck.[165, 167, 168] He correlated the degree of leukergy of rabbit leukocytes with the alkaline phosphatase and glycogen contents of the cells.[168] Leukergic leukocytes apparently clump in normal plasma, but normal leukocytes do not necessarily clump in plasma from patients whose leukocytes show the leukergic phenomenon. The reaction was therefore thought to be cellular. Tischendorf and Fritze [520] also believed that leukergy is due to cell factors rather than plasma factors. Hartman, however, presented evidence possibly suggesting that leukergy is not due to inherent cell changes but is associated with the water-soluble serum globulin fraction of the blood of animals with inflammation.[217] Likewise, Schaper's evidence might support the plasmatic origin of a form of leukergy.[445] It may well be that a number of mechanisms are involved, only one of which is cellular and might properly be called "leukergy." In any case it may be said for our purposes that leukergy might interfere with immunologic work on the leukocytes of acute infection or other stress states, especially if agglutination tests are contemplated.

Selective Destruction of Red Blood Cells

A number of procedures lead to destruction of red cells without destroying leukocytes. These procedures involve the use of either gramicidin and lysolecithin,[477] saponin,[453] an alcohol-water mixture,[155] anti-A or anti-B hemolytic serum,[104] an acetic acid-tartaric acid mixture [412, 457, 511, 512] or distilled water followed by concentrated buffered salt solution.[543] The preparation of the gramicidin and lysolecithin reagents is quite complicated, and the method has not as yet been used for immunologic work. Saponin causes too much damage to leukocytes to be a suitable agent, according to Wasastjerna's experience [545] as well as our own. No immunologic studies have been done with the alcohol-water method. The use of anti-A or anti-B hemolytic serum against group A or B blood raises the problem of erythrophagocystosis and mixed clumping, as indicated by Butler [72] and Bakemeier and Swisher,[23] so it is probably unsuitable.

Among the methods in this category the acetic acid-tartaric acid procedure and the distilled water-concentrated buffer procedure are

preferable. The former is based on the observation that while HAc or tartaric acid alone are highly injurious to white blood cells, in combination they are very much less so; and the latter, that red blood cells are hemolyzed much sooner than leukocytes on exposure to hypotonic solutions. While we have employed suspensions prepared by these methods in performing leuko-agglutinin tests, the results have been somewhat variable and not consistently satisfactory. For other immunologic tests the methods may, however, be useful, for leukocyte material of high purity can be obtained.

Szilard's original acetic acid-tartaric acid method,[511, 512] published first in 1923, was for whole blood. By using leukocyte-rich supernatant from sedimented blood, the concentration of the reagents can be cut down. I have employed the following modification with fair success. Reagents: 2½ per cent HAc, 2 per cent tartaric acid (made up fresh because easily contaminated), 2 per cent KOH and 0.85 per cent saline. Mix 6 ml. of the HAc with 1.5 ml. of the tartaric and add the mixture to 10 ml. of supernatant from sedimented blood. When hemolysis is complete by visual examination, add a few drops of phenolphthalein indicator, then KOH to neutrality. Then add an additional 0.7 ml. of KOH. Centrifuge five to seven minutes at 500 rpm, wash once with saline and resuspend in normal serum or saline. If the supernatant from the first centrifugation is cloudy or brown, one has waited too long before adding the KOH or else an inadequate amount has been added, and the preparation is useless. Both Locke's [512] and Tyrode's [412] solutions have been substituted for the saline; but Seabright [457] found that Locke's solution caused more nonspecific clumping than saline. Szilard [512] stated that the leukocytes prepared by his method showed active ameboid motility. This has also been our experience. It may be pointed out, however, that while Hirsch [231] confirmed the ameboid motility of leukocytes separated by Szilard's method, he found their respiration as measured in a Warburg apparatus adversely affected. This recalls Tullis' statement that in descending order a separated leukocyte loses its oxidative metabolism, phagocytic ability, ameboid motility, resistance to impermeable dyes, Brownian movement and morphologic integrity.[522, 524]

The distilled water-concentrated salt method is as follows. Centrifuge 10 ml. of leukocyte-rich plasma for seven minutes at 500 g. Leukocytes and erythrocytes are thereby thrown to the bottom of the tube. Remove all supernatant. Rapidly blow 6 ml. of distilled water into the tube, resuspend all cells by agitation and in exactly 25 seconds blow in 2 ml. of a 3.4 per cent solution of buffered saline. Recentrifuge, remove the pink supernatant and resuspend in saline or plasma. Manipulation with a Pasteur pipette will break up most of the nonspecific clumping. The procedure can be repeated if not all red blood cells have been hemolyzed. Lapin et al.[279] reported considerable loss in ameboid motility of leukocytes separated by a method similar to that given above.

In Fiessinger's alcohol-water method,[155] 10 ml. of fresh blood were placed immediately into 50 ml. of a one-third alcohol, two-thirds water solution, centrifuged rapidly and the supernatant decanted. The button so obtained was said to be a