Biophysical Chemistry 5 (1976) 99–106

SEPARATION OF TUMOR CELLS BY DENSITY GRADIENT CENTRIFUGATION: RECENT WORK WITH HUMAN TUMORS AND A DISCUSSION OF THE KIND OF QUANTITATION NEEDED IN CELL SEPARATION EXPERIMENTS*‡

Thomas G. PRETLOW II, C. Michael JONES and Theresa P. PRETLOW
Departments of Pathology and Engineering Biophysics, University of Alabama Medical Center, Birmingham, Alabama 35294, USA

The application of centrifugation to the purification of individual kinds of cells from tumors has been reviewed. Recent applications of a previously described isokinetic gradient to the purification of subpopulations of cells from human tumors are presented. Individual kinds of cells have been separated from Hodgkin's disease by a method described previously, and the cells from Hodgkin's disease which contain the tumor associated antigen described by Order and his colleagues have been identified for the first time. The importance of the thorough documentation of cell separation procedures is discussed, and we have specifically listed several kinds of data which we believe are important in the documentation of cell separation techniques.

1. Introduction

In the investigation of specific kinds of cancer cells and their specific, ancestral, precursor cell types, quantitative characterization is made more difficult by the fact that both the neoplastic cells in cancers and their ancestral precursors in normal tissues are frequently diluted by large numbers of other kinds of cells. When enzymes from a carcinoma are found to be kinetically different from the corresponding enzymes in the normal tissue from which the carcinoma developed [1], one wonders if these differences reflect differences between the malignant cell and its ancestral precursor or simply differences in the frequency distribution of nonepithelial, stromal cell types. Sanders and his associates [2] have expressed the view that the unavailability of pure cell types from tumors is an important difficulty which has "beset all attempts to implicate particular viruses as a cause of human tumors". In addition to viral oncologists, many investigators who are interested in the culture of tumors have commented on the common problem that fibroblasts and undesired cell types often overgrow cultures of mammalian tumors [3–5]. Like the malignant cells from tumors, stromal cells and cells which invade the tumor as a part of the host's defense against cancer are being characterized in vitro by cancer researchers. When stromal or lymphoid cells are observed to kill tumor cells in vitro, our interpretation of this phenomenon must be limited if the target tumor cells and/or the attacking "killer" cells are diluted by variable, often unspecified proportions of other kinds of cells. In years past, it would appear that many experimental oncologists routinely ignored the fact that tumors contain large numbers of lymphocytes, histiocytes, fibroblasts, blood cells, and cells which form the blood vessels. In recent years, there have been numerous attempts to characterize and/or purify both the malignant cells and other stromal [6–14] cell types from tumors. In this symposium, we shall briefly review the reported separations of both malignant and stromal cell types from tumors using centrifugation. We shall

* Supported by grants from the National Institutes of Health (CA-16764, CA-15089, CA-16430, and DE-2670) and the American Cancer Society (DT-43A). Dr. Pretlow is supported by NIH Research Career Development Award K4-CA-70584-03.
‡ Address reprint requests to Dr. Thomas G. Pretlow II, Department of Pathology, University of Alabama in Birmingham, University Station, Birmingham, Alabama 35294.

then present some of our recent applications of centrifugation to the purification of cells from human tumors; and we shall conclude with some thoughts about one of the most critical problems in the general area of cell separation: the establishment of criteria which should be applied to the evaluation of cell separation experiments.

2. Separation of tumor cells by centrifugation

Central to any rational attempt to separate tumor cells by centrifugation is the recognition that cells possess only two physical properties, diameter and density, which are useful in the design of experiments for their purification by centrifugation. The theory of gradient centrifugation as related to the separation of mammalian cells has been treated in detail elsewhere [15–20], and will, therefore, not be treated in detail here.

Early attempts to separate cells from tumors by centrifugation were carried out with neutral density columns. In 1950, Fawcett et al. [21,22] reported the modification of a neutral density technique, which they had employed previously in work with blood cells [23], for the purification of malignant cells from pleural and ascites fluids. In their work, malignant cells, mesothelial cells, and histiocytes were retained at the sample–albumin interface, while red blood cells, leukocytes, and debris passed through albumin at a concentration sufficient to give a density between 1.05 and 1.07 gm/ml. They recognized the potential diagnostic utility of their technique and suggested that refinement of their technique might result in preparations of malignant cells of sufficient purity to be useful for the chemical and physical characterization of malignant cells.

A modification of the neutral density technique was subsequently employed by Roberts et al. [24] for the separation of malignant cells from the peripheral blood of patients with cancer. A decade following the first neutral density separation of tumor cells by Fawcett et al. [21], Spriggs and Alexander [25] noted that: "When a variety of cells together with platelets are packed at an interface they are apt to sink or float as a unit. Such artefacts may seriously interfere with separation...". They found that, if they stirred the interface between the sample and the neutral density layer of albumin, these artifacts were less limiting, and they obtained three layers of cells. The top layer contained lymphocytes, platelets, and monocytes. In some cases, this layer was split into two layers, one of which was relatively pure platelets. The middle layer contained granulocytes, and the bottom layer contained mostly red blood cells. When present, tumor cells were generally found among the monocytes, lymphocytes, and/or platelets. It is interesting to note that the order of distribution of the blood cells and platelets which they obtained closely approximates that found following isopycnic centrifugation in continuous, linear gradients of Ficoll in tissue culture medium [26]. Seal [27] performed similar separations of tumor cells from peripheral blood by neutral density flotation over silicone.

In the 1940s, 1950s, and early 1960s, the use of a neutral density column or a discontinuous gradient (in effect a series of neutral density columns) was a progressive idea. In the 1970s it is surprising to find some investigators who continue to use neutral density columns and discontinuous gradients for the separation of tumor cells [28–30]. A few years ago, de Duve [31] stated: "The discontinuous gradient is essentially a device for generating artificial bands. This may be a convenient way of compressing together for preparative purposes certain segments of the distributions observed in continuous gradients. But it is also a very dangerous procedure in that it creates the illusion of clear-cut separation." Recent reviews by Leif [32], Shortman [33], and Pretlow II et al. [20] have emphasized the limitations which are inherent in the use of discontinuous gradients for the separation of cells.

Most separations of tumor cells in continuous gradients have been carried out by isopycnic centrifugation in colloidal silica [34], Renografin 60 (methylglucamine N,N′-diacetyl-3,5-diamino-2,4,6-triiodobenzoate) [35], bovine serum albumin [36], or Ficoll (polysucrose, average molecular weight of 400 000) [16,37–41]. In some reports, there was no published evidence that the authors were aware that tumors contain cell types that are not malignant other than blood cells. In general, there are few examples of the separation of tumor cells in which isopycnic centrifugation is as effective as velocity sedimentation. In work with a mast cell tumor, Pertoft [34] obtained some fractions in which "practically all [cells] contained metachromatic granules". If we assume that these granules were not the ingested products of other cells, most of

the cells in these purified populations must have been mast cells. The efficacy of isopycnic centrifugation for the purification of malignant mast cells is not unexpected in view of the fact that normal mast cells [42] can be highly purified by isopycnic centrifugation; in this respect, mast cells differ from most other kinds of normal cells. Isopycnic centrifugation has been applied to the separation of various cell types from mast cell tumors [34]; human leukemias [36,38]; mouse ascites tumors [16,39]; and other mouse transplantable tumors [37,40].

Despite the rapidly increasing volume of work on the purification of cells from animal tumors by centrifugation, there has been little work on the purification of cells from human solid tumors by isopycnic or velocity gradient centrifugation. To our knowledge, the only published, well-documented separation of specific cell types from human tumors with a high degree of purity was the report from our laboratory of the purification of different cell types from the tumor of Hodgkin's disease [43]. We have published our purification of epithelial cells from hamster [44] and human [45] prostates and will soon publish our purification of epithelial cells from human prostatic carcinomas. Except for the rather unusual case of malignant, rodent mast cells [34], the densities of malignant cells and other tumor cells from cancers overlap broadly; consequently, isopycnic centrifugation is not as useful as velocity sedimentation for the separation of specific kinds of cells from tumors.

There have been few published comparisons of isopycnic sedimentation with velocity sedimentation as methods for the separation of individual kinds of cells from tumors. When such comparisons have been made, with one exception, velocity sedimentation has proved to be the more useful of the two modalities. The exception to which we refer is the report by Abeloff et al. [38] in which various cell types from human leukemic blood were separated with approximately equal efficacy by velocity and by isopycnic centrifugation. This work was performed and published prior to the development of an isokinetic gradient [19] which we have found to be capable of superior resolution when compared to earlier gradients for the separation of cells by velocity sedimentation. Several kinds of cells have been highly purified from solid tumors by velocity sedimentation in gradients of Ficoll in tissue culture medium [37,39,40,43,45]. Lindahl and his associates [46–48] have described the separation of different kinds of cells from ascites tumors by velocity sedimentation using a special device and technique which have been reviewed recently [20].

3. Recent application of centrifugation to the purification of tumor cells

As discussed above, purification of malignant cells from tumors presents the opportunity to compare malignant cells with their normal, ancestral, precursor cell types. One can also characterize individual kinds of purified stromal cells. In addition, we stumbled upon some applications which were not anticipated but which have been very useful. Early in 1974, we were sent a pleural effusion for diagnostic evaluation. The pathologist who had performed the routine examination of the fluid from this effusion previously had noted that the effusion contained highly undifferentiated singly suspended cancer cells; however, with the available material, he was unable to be more specific. We examined the fluid and arrived at the same conclusion; however, because there were so many interesting looking tumor cells which appeared viable, we froze them viably as described previously [43] for cells from Hodgkin's disease in order to attempt to culture them at a later date. A few days later, one of the medical students who was doing an elective in the laboratory asked if he could see how they sedimented in the isokinetic gradient [19]. To our surprise, following sedimentation we noted a few, small, well-defined adenoid structures in the fraction at the gradient–cushion interface. These structures were diluted in the unseparated suspension by more than 99.9% single cells (predominantly blood cells) and had sedimented more rapidly than most other cells because of the large "effective diameter" [17] which they exhibited collectively as an aggregate. On returning to follow the progress of the patient, we found that he had had surgery one day prior to our cell separation experiment, and the biopsy of the tumor had demonstrated that it was an adenocarcinoma. Had we been able to observe the adenoid structures in the unseparated cells (fig. 1) which were obvious in the concentrated fraction (fig. 2) at the bottom of the gradient, the diagnosis would have been made without the surgical biopsy.

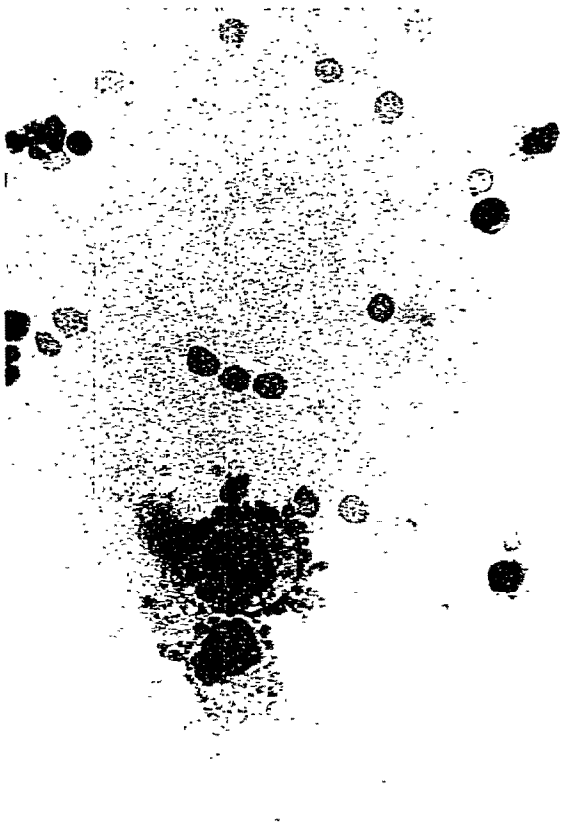

Fig. 1. Unseparated cells from a malignant pleural effusion. Prior to purification, the most frequently observed cells were blood cells and occasional, anaplastic tumor cells (Wright stain × 250).

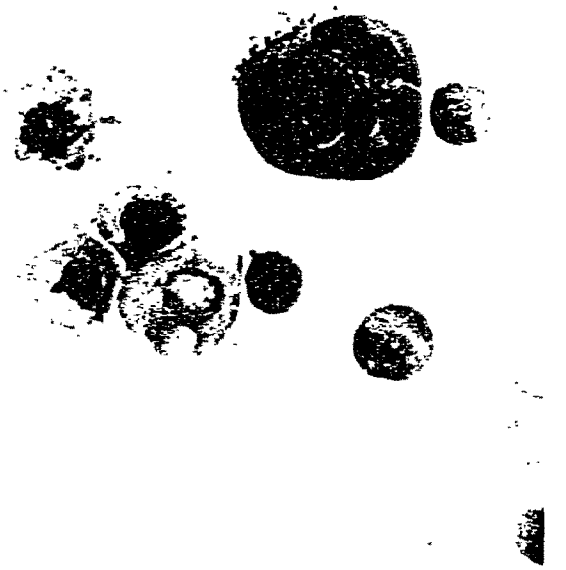

Fig. 2. Cells at the gradient–cusion interface following velocity sedimentation of cells from the malignant pleural effusion shown in fig. 1. Well-defined adenoid structures are seen which permit the diagnosis of adenocarcinoma (Wright stain × 250).

Similar gradient separations have resulted in the concentration of tiny subpopulations of cells which had been overlooked from many different sources. One of the most exciting of these findings which will be published in detail in the future [49] is the identification of the cells from Hodgkin's disease which contain the Hodgkin's disease associated antigen described by Order and his associates [50–56]. This antigen has been highly purified by them from the tumor of Hodgkin's disease and has been localized to the tumor by immunofluorescence; however, they were not able to identify the cell types which possessed the Hodgkin's associated antigen. Initially, in work with unseparated cell suspensions, we found very few cells which were antigenic and did not recognize any pattern which distinguished cells with antigen from cells without antigen. After separation in the isokinetic gradient, the fraction at the gradient–cushion interface, which contained 1–3% of the cells in the suspension prior to separation, was found to contain almost all of the antigenic cells. Characteristically, these cells were arranged in rosettes which appeared to consist of large, centrally located atypical histiocytes or Reed–Sternberg cells about which small, intensely antigenic lymphoid cells were rosetted (figs. 3 and 4).

We have recently studied several isoenzyme systems in purified cancer cells [57] and expect that, as suggested by Fishman [58], the availability of purified preparations of single kinds of cells will do much to resolve some of the problems in the study of isoenzymes in cancer.

Fig. 3. Bright field photomicrograph of typical antigenic cells in rosettes purified from suspensions of cells from the tumor of Hodgkin's disease. Typically, only a small minority of the cells from Hodgkin's disease contain the tumor associated antigenic activity described by Order. When stained using rabbit antibody which was generously supplied by Dr. Order and a secondary, peroxidase labeled, goat antirabbit gammaglobulin, the most intensely antigenic cells were small lymphocytes which were found in rosettes adherent to larger, centrally located, less intensely antigenic, atypical histiocytes and Reed–Sternberg cells. The antigenic rosettes were separated from the majority of cells from Hodgkin's disease and were located in fractions which contained only 1–3% of the cells separated in the isokinetic gradient. Less than 5% of lymphocytes from the tumor showed antigenic activity. The conditions for the gradient separation are the same as described prior [43] to our identification of the cells with tumor associated antigens (stained to demonstrate peroxidase labeled antibody with methyl green counterstain × 200).

Fig. 4. Phase photomicrograph of same cells shown in fig. 3 (× 200).

4. Criteria for the evaluation of cell separation experiments

In our view, there are two critical obstacles which retard progress in the investigation of purified cells. The first of these obstacles is the lack of good methods for obtaining some kinds of cells in suspension. The available methods for obtaining suspensions of cells from solid tissues have been reviewed recently [20] and will not be treated here. The other major obstacle to progress in cell separation is the absence of a widely accepted concept of the kinds of data which are necessary to document experiments in cell separation. In discussing a somewhat similar area of investigation, de Duve [31] wrote: "...in the early days of centrifugal fractionation, adequate purification of a subcellular organelle was quite unattainable technically, and there were practically no means of evaluating the purity of a preparation. This is how, for instance, nuclei came to be credited with such a wealth of cytoplasmic enzyme activities ... Even today, with all our technical improvements, and with the vast advances in our knowledge, preparative fractionation remains a hazardous undertaking." Many of the best journals in biochemistry and immunology are currently publishing very sophisticated studies of cells which the authors

call "purified" with no numerical expression of purity and with virtually no critical attention to the *criteria* which the author employed to assess purity. One wonders how the same editors would react to a submitted manuscript describing the kinetics or amino acid sequence of a newly "purified" enzyme in the absence of any attempt to describe the extent of the purification.

If we are to characterize a biochemical or immunological function of a purified cell from a solid tissue, it would seem desirable that we attempt to assess how well the separated cell reflects the function of the cell prior to separation. Ideally, it would seem desirable that morphometric studies [59–64] be performed which permit one to compare the quantity of each cell type in the intact tissue and in the cell suspensions obtained from the tissue prior to cell separation. While morphometric studies may not be feasible in all laboratories, as a very minimum, the investigator should inform the reader of the number of each type of cell which he obtained in suspension per gram of tissue wet weight. This data is usually omitted from papers which describe cell separation; and, without this data, it is impossible for an investigator who wishes to repeat the experiment to a make a meaningful comparison between his unseparated cells and those in the original report. Moreover, without some knowledge of the frequency of distribution among the cells prior to separation, it is impossible for the reader or the author to know whether any real purification took place during the cell separation experiment. In addition to giving both the absolute number of cells obtained and the relative frequency of each cell type, the investigator should indicate the variability which he encountered among experiments. In surveying work which has been carried out in cell separation [20], one is led to suspect that many investigators who separate cells do not believe that confidence intervals or standard deviations are useful. Complex experiments are carried out and important concepts are obtained from the passive transfer of purified cells which are typically described as "enriched for PB cells [specificity-restricted precursors of B cells] and stem cells but nearly free of B cells..." [65]; we are not told how much enriched, how nearly free, *or what other kinds of cells were present* among the passively transferred cells "enriched for PB cells and stem cells". The importance of the numerical description of purity and of a critical analysis of the data base has been emphasized in a recent report from a WHO/IARC-sponsored workshop which discussed the "identification, enumeration, and isolations" of various kinds of immunologically active cells [66].

In the absence of precise identification and enumeration of cells both before and after cell separation experiments, it is quite obviously impossible to calculate recovery. Without the quantitative calculation of recovery, a procedure which is routinely omitted in some laboratories, it is difficult to arrive at any meaningful evaluation of experiments which employ techniques for the separation of cells. As de Duve [31] has recently discussed the importance of "...quantitative recovery and analysis of all fractions, the hallmark of analytical fractionation", we shall not dwell on this very important topic.

Having assessed the extent of the purification and the recovery, the investigator should attempt to evaluate the function of the cells both before and after separation. With this data, it should be possible (a) to evaluate the effect, if any, of the purification procedure on cell function and (b) to compare the function of the purified cells with the function of the intact tissue. In addition to measuring the collective function of purified cells from specific fractions, the investigator should attempt to evaluate some functions of cells individually. The collective functions of cells in individual fractions can be assessed in many different ways: passive transfer to transplant recipients, biochemical assays, etc.. Similarly, the function of individual cells can be evaluated by dye exclusion, light and electron microscopy, labeled antibodies to cytoplasmic components, etc.. We wish to emphasize the fact that assays of collective function give a *kind* of information which is qualitatively different from that obtained from assays of the function of individual cells; both kinds of information seem valuable.

In our view, photomicrographs of permanent preparations are among the most important kinds of data which are omitted from most reports of cell separation. Photomicrographs of permanent preparations allow the reader to evaluate the morphologic integrity of individual cells and to determine whether or not the morphologic criteria used for cell identification were adequate. Photomicrographs should be taken at a magnification which will permit a considerable sample of the cells to be evaluated by the reader and which

will give sufficient morphologic detail to be meaningful. In addition, the reader will be able to judge whether or not the "purified" cells are single or aggregated. Permanent preparations have the advantage over wet preparations that more cytologic detail is visible. In addition, permanent preparations are easily stored and provide an important part of the permanent record of the experiment.

During the process of separating cells, fractions should not be so large that a "peak" of purified cells is encompassed in a single fraction. In a recent report [67], megakaryocytes were separated into three subpopulations by velocity sedimentation. In the absence of any knowledge of the respective densities of these cells, the authors assigned sizes (6940, 14040, and 31770 μm^3) to these subpopulations based upon their separation by velocity sedimentation over a total of seven gradient fractions, i.e., there were only seven available categories with respect to velocity of sedimentation. Aside from assigning diameters to these subpopulations (of unknown density) with four significant figures, the authors identified one of these subpopulations by virtue of the fact that it was contained in a peak which was represented by a single data point. In our view, except when peaks are artifically sharpened at the gradient–cushion interface, *"peaks" should contain more than one data point*, and this end can be accomplished simply by adjusting the sizes of the fractions from the gradients.

Before concluding, we should mention the dangers which are inherent in total reliance upon electronic cell counters for work with cell suspensions from solid tissues. In our experience, electronic cell counters cannot distinguish cells from debris. Despite the use of window discriminators, aggregates of small cells are counted as though they were equivalent to single large cells; and large subcellular particles, e.g., tetraploid hepatic nuclei, are counted as cells. Other problems which are inherent in the use of electronic cell counters have been discussed in previous reports [68–71]. While electronic cell counters are more useful for some kinds of cells than for others, they should always be evaluated for any particular kind of cell suspension by an independent method of counting cells.

We recognize that some of the views expressed here will not be unanimously heralded with total agreement and hope that we shall stimulate a critical discussion of the kinds of data which are necessary in the evaluation of cell separation experiments.

References

[1] M.I. Ringler and R. Hilf, Enzyme 18 (1974) 257.
[2] M. Eisinger, S.M. Fox, E. De Harven, J.L. Biedler and F.K. Sanders, Nature 233 (1971) 104.
[3] D.J. Giard, S.A. Aaronson, G.J. Todaro, P. Arnstein, J.H. Kersey, H. Dosik and W.P. Parks, J. Natl. Cancer Inst. 51 (1973) 1417.
[4] A.J. Dalton, U. Heine, J.M. Kondratick, D.V. Ablashi and E.A. Blackham, J. Natl. Cancer Inst. 50 (1973) 879.
[5] L.A. Cohen, J. Tsuang and P.C. Chan, In Vitro 10 (1974) 51.
[6] A. Richters and R.P. Sherwin, Cancer 34 (1974) 328.
[7] S. Brem, R. Cotran and J. Folkman, J. Natl. Cancer Inst. 48 (1972) 347.
[8] M.S.C. Birbeck and R.L. Carter, Int. J. Cancer 9 (1972) 249.
[9] J.C.E. Underwood, Br. J. Cancer 26 (1972) 234.
[10] G.T. Diamandopoulos and M.F. Dalton-Tucker, Amer. J. Pathol. 56 (1969) 59.
[11] I.F. Tannock and G.G. Steel, J. Natl. Cancer Inst. 42 (1969) 771.
[12] J. Kawamura, J.H. Garcia and Y. Kamijyo, Cancer 31 (1973) 1528.
[13] G. Burg and O. Braun-Falco, Arch. Derm. Forsch. 245 (1972) 318.
[14] M. Paavolainen, J. Tarkkanen and E. Saksela, Acta Otolaryng. 75 (1973) 316.
[15] C.W. Boone, G.S. Harell and H.E. Bond, J. Cell Biol. 36 (1968) 369.
[16] T.G. Pretlow II and C.W. Boone, Science 161 (1968) 911.
[17] T.G. Pretlow II and C.W. Boone, Exptl. Mol. Pathol. 11 (1969) 139.
[18] T.G. Pretlow II, C.W. Boone, R.I. Shrager and G.H. Weiss, Anal. Biochem. 29 (1969) 230.
[19] T.G. Pretlow, Anal. Biochem. 41 (1971) 248.
[20] T.G. Pretlow II, E.E. Weir and J.G. Zettergren, in: International review of experimental pathology, Vol. 14, eds. G.W. Richter and M.A. Epstein (Academic Press, New York, 1975) pp. 91–204.
[21] D.W. Fawcett, B.L. Vallee and M.H. Soule, Science 111 (1950) 34.
[22] D.W. Fawcett and B.L. Vallee, J. Lab. Clin. Med. 39 (1952) 354.
[23] B.L. Vallee, W.L. Hughes and J.G. Gibson, Blood, Spec-Issue 1 (1947) 82.
[24] S. Roberts, A. Watne, R. McGrath, E. McGrew and W.H. Cole, A.M.A. Arch. Surg. 76 (1958) 334.
[25] A.I. Spriggs and R.F. Alexander, Nature 188 (1960) 863.
[26] T.G. Pretlow II, unpublished data.
[27] S.H. Seal, Cancer 12 (1959) 590.

[28] J.E. de Vries, M. van Benthem and P. Rümke, Transplantation 15 (1973) 409.
[29] J.A. Sykes, J. Whitescarver, L. Briggs and J.H. Anson, J. Natl. Cancer Inst. 44 (1970) 855.
[30] R.B. Livingston, U. Ambus, S.L. George, E.J. Freireich and J.S. Hart, Cancer Res. 34 (1974) 1376.
[31] C. de Duve, J. Cell Biol. 50 (1971) 20d.
[32] R.C. Leif, in: Automated cell identification and cell sorting, eds. G.L. Wied and G.F. Bahr, (Academic Press, New York, 1970) pp. 21–96.
[33] K. Shortman, in: Annual review of biophysics and bioengineering, Vol. 1, ed. M.F. Morales (Annual Reviews Inc., Palo Alto, California, 1972) pp. 93–130.
[34] H. Pertoft, J. Natl. Cancer Inst. 44 (1970) 1251.
[35] D.J. Grdina, L. Milas, K.A. Mason and H.R. Withers, J. Natl. Cancer Inst. 52 (1974) 253.
[36] R.M. Zucker and B. Cassen, J. Natl. Cancer Inst. 52 (1974) 1691.
[37] T.G. Pretlow II and C.W. Boone, Exptl. Mol. Pathol. 12 (1970) 249.
[38] M.D. Abeloff, R.J. Mangi, T.G. Pretlow and M.R. Mardiney, J. Lab. Clin. Med. 75 (1970) 703.
[39] M.J. Stewart, T.G. Pretlow II and R. Hiramoto, Amer. J. Pathol. 68 (1972) 163.
[40] J.G. Zettergren, D.E. Luberoff and T.G. Pretlow II, J. Immunol. 111 (1973) 836.
[41] R.L. Warters and K.G. Hofer, Exptl. Cell Res. 87 (1974) 143.
[42] T.G. Pretlow II and I.M. Cassady, Amer. J. Pathol. 61 (1970) 323.
[43] T.G. Pretlow II, D.E. Luberoff, L.J. Hamilton, P.C. Weinberger, W.A. Maddox and J.R. Durant, Cancer 31 (1973) 1120.
[44] S.H. Dow and T.G. Pretlow II, J. Natl. Cancer Inst. 54 (1975) 147.
[45] S.R. Helms, F.I. Brazeal, A.J. Bueschen and T.G. Pretlow II, Amer. J. Pathol. 80 (1975) 79.
[46] L. Sörenby and P.E. Lindahl, Exptl. Cell Res. 35 (1964) 214.
[47] P.E. Lindahl, Cancer Res. 20 (1960) 841.
[48] P.E. Lindahl and G. Klein, Nature 176 (1955) 401.
[49] T.G. Pretlow II and E.E. Weir, in preparation.
[50] S.E. Order, M. Porter and S. Hellman, N. Eng. J. Med. 285 (1971) 471.
[51] S.E. Order, S.E. Chism and S. Hellman, Blood 40 (1972) 621.
[52] S.E. Chism, S.E. Order and S. Hellman, Amer. J. Roent. 117 (1973) 5.
[53] D.H. Katz, S.E. Order, M. Graves and B. Benacerraf, Proc. Natl. Acad. Sci. 70 (1973) 396.
[54] S.E. Order, J. Colgan and S. Hellman, Cancer Res. 34 (1974) 1182.
[55] S. Order, S. Chism and S. Hellman, Natl. Cancer Inst. Monog. 36 (1973) 139.
[56] S.E. Order and S. Hellman, J. Amer. Med. Assoc. 223 (1973) 174.
[57] S.R. Helms, Thesis, University of Alabama in Birmingham (1975).
[58] W.H. Fishman and F. Lerner, J. Biol. Chem. 200 (1953) 89.
[59] E.R. Weibel, W. Stäubli, H.R. Gnägi and F.A. Hess, J. Cell Biol. 42 (1969) 68.
[60] F.R. Robinson, H.W. Casey and E.R. Weibel, Amer. J. Pathol. 76 (1974) 175.
[61] G.S. Kistler, P.R.B. Caldwell and E.R. Weibel, J. Cell Biol. 32 (1967) 605.
[62] E.R. Weibel, G.S. Kistler and W.F. Scherle, J. Cell Biol. 30 (1966) 23.
[63] W. Stäubli, R. Hess and E.R. Weibel, J. Cell Biol. 42 (1969) 92.
[64] O. Greengard, M. Federman and W.E. Knox, J. Cell Biol. 52 (1972) 261.
[65] R.A. Phillips and R.G. Miller, Nature 251 (1974) 444.
[66] F. Aiuti, J.-C. Cerottini, R.R.A. Coombs, M. Cooper, H.B. Dickler, S.S. Froland, H.H. Fudenberg, M.F. Greaves, H.M. Grey, H.G. Kunkel, J.B. Natvig, J.-L. Preud'homme, E. Rabellino, R.E. Ritts, D.S. Rowe, M. Seligmann, F.P. Siegal, J. Stjernswärd, W.D. Terry and J. Wybran, Scand. J. Immunol. 3 (1974) 521.
[67] A. Nakeff and B. Maat, Blood 43 (1974) 591.
[68] B.S. Bull, Blood 31 (1968) 503.
[69] R.J. Harvey, in: Methods in cell physiology, Vol. 3, ed. D.M. Prescott (Academic Press, New York, 1968) pp. 1–23.
[70] K. Shortman, J. Cell. Physiol. 77 (1971) 319.
[71] A.C. Groom and J.C. Anderson, J. Cell. Physiol. 79 (1972) 127.