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A Method for the Separation of Lymphocytes and Plasma Cells from the Human Palatine Tonsil Using Sedimentation in an Isokinetic Gradient of Ficoll in Tissue Culture Medium

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Summary. Several methods for the dissociation of human tonsils into cell suspensions were compared. Dissociation of tonsils using 0·25 per cent trypsin gave both the largest number of total cells and the largest number of plasma cells per gram of tonsil. Lymphocytes and plasma cells were separated in a previously described isokinetic gradient of Ficoll in tissue culture medium. In the purest gradient fractions, lymphocytes were 97·2±1·9 per cent of nucleated cells. The purest gradient fractions contained 43·1±5·9 per cent plasma cells. More than 95 per cent of purified lymphocytes and plasma cells excluded Trypan Blue.

INTRODUCTION

Numerous methods have been developed for the separation of individual kinds of cells from the peripheral blood; these methods have been reviewed recently (Pretlow, Weir and Zettergren, 1974b; Shortman, 1972). While lymphocytes from bone marrow, spleens, and lymph nodes have proved very valuable in the investigation of the immune systems of experimental animals, most *in vitro* studies of human lymphocytes have been conducted using lymphocytes from the peripheral blood. Its convenient availability has been an important factor in the relative emphasis which has been given to the lymphocyte from human peripheral blood. Because of the enormous differences which have been found between lymphocytes from different lymphoid tissues in experimental animals, one would anticipate that much could be learned from the development of methods which would permit the purification of single kinds of cells for study from other human lymphoid tissues. The human palatine tonsil is readily available in the operating rooms of most general hospitals. In this report, we compare several methods for obtaining cell suspensions from human tonsils and describe a method for separating lymphocytes and plasma cells from suspensions of human tonsillar cells using velocity sedimentation.

The method which we have employed for the separation of lymphocytes and plasma cells from the tonsil has been employed previously for the purification of other human lymphocytes including lymphocytes from human peripheral blood (Pretlow and Luberoff,

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1973) and lymphocytes from the tumour of Hodgkin's disease (Pretlow, Luberoff, Hamilton, Weinberger, Maddox and Durant, 1973). Other applications of this method for cell separation, including the purification of lymphocytes from disaggregated transplantable tumours (Zettergren, Luberoff and Pretlow, 1973), have been reviewed recently (Pretlow *et al.*, 1974b). Following purification, more than 90 per cent of cells have excluded Trypan Blue in all examined systems. Separated cardiac myocytes have continued to contract with an intrinsic autonomous rhythm (Pretlow, Glick and Reddy, 1972); bone marrow cells which have the capacity to form spleen colonies have been grown in irradiated, syngeneic transplant recipients; cells from the proximal tubule of the hamster kidney have been cultured for several months (Pretlow, Jones and Dow, 1974a), and tumour cells have been grown both in culture and in syngeneic transplant recipients (Pretlow and Boone, 1968; Stewart, Pretlow and Hiramoto, 1972).

Cells that differ with respect to diameter are best separated by velocity sedimentation. We have found a previously described isokinetic gradient (Pretlow, 1971) to be the most broadly applicable gradient for the separation of cells by velocity (rate-zonal) sedimentation. In contrast to the gradients (Pretlow and Cassady, 1971; Pretlow *et al.*, 1972) that we employed prior to the development of the isokinetic gradient, no computer is required for the design of experiments carried out using the isokinetic gradient. We now report a one-step method for the separation of lymphocytes and plasma cells from suspensions of cells from the human tonsil. While neither cell type is obtained in absolute purity, this is the first report, to our knowledge, of a procedure which permits the separation of the modal population of lymphocytes from the modal population of plasma cells. We discuss the available cell separation methods which might be employed in sequence with the described method in order to obtain subpopulations of more highly purified cells.

MATERIALS AND METHODS

Previous reports contain detailed descriptions of the methods (Pretlow and Boone, 1969; Pretlow *et al.*, 1974b), theory (Boone Harell and Bond, 1968; Pretlow and Boone, 1968; Pretlow, 1971; Pretlow *et al.*, 1974b), and a survey of the previous cell separations (Pretlow *et al.*, 1974b) which have been accomplished in this laboratory using gradients of Ficoll (polysucrose, average mol. wt 400,000, Pharmacia Fine Chemicals, Piscataway, New Jersey) in tissue culture medium. We shall therefore omit a detailed description of the methods from this report.

Cell suspensions

Before studying the actual separation of individual kinds of cells from the tonsil, we investigated several methods for obtaining the cells from tonsils in suspension. Tonsils were obtained in the operating room, and were placed in cold tissue culture medium immediately after being removed from the patients. Sections were taken for the surgical pathologist; adherent blood clots were dissected free; and the remaining tissue was weighed, divided into three equal portions, and minced to fragments measuring 1–2 mm in maximum dimension. Each of the three aliquots of minced tonsil was washed for five 10-minute periods in separate, 40-ml changes of Joklik's modification of minimum essential medium containing 10 per cent foetal calf serum (both from Grand Island Biological Company, Grand Island, New York). During the washing procedure, the fragments were kept in suspension by gentle agitation on a magnetic stirrer. The washing procedure removed

many red blood cells as well as some nucleated cells. Preliminary studies using mincing, teasing, and other physical methods for obtaining cell suspensions (Harris and Harris, 1954; Helmreich, Kern and Eisen, 1961; Malmgren, 1958; Roberts and Dixon, 1955; Vann and Kettman, 1972) resulted in our obtaining consistently low yields of cells per gram of tissue including a high proportion of cells which failed to exclude Trypan Blue.

After washing, the three aliquots of minced tonsil were digested separately and simultaneously with 0·05 per cent Sigma Type II collagenase (Sigma Chemical Company, St Louis, Missouri) in Joklik's modification of minimum essential medium, 0·1 per cent pronase (EM Laboratories, Elmsford, New York) in culture medium without serum, and 0·25 per cent trypsin (Grand Island Biological Company) in culture medium without serum. Digestion was accomplished using thirteen successive 20-minute digestion periods during which the tissue fragments were maintained in suspension at 22° (room temperature) using a magnetic stirrer. At the end of each 20-minute digestion period, the tissue fragments were allowed to settle; the supernatant suspensions of cells were decanted and chilled in an ice bath, and 40 ml of fresh enzyme solution was added to each flask. After chilling for 5 minutes, the cells were sedimented at 90 ***g*** for 7·5 minutes, the used enzyme solution was discarded, and the cells were suspended in five volumes of culture medium containing 10 per cent foetal calf serum. After the tissue had been exhaustively digested, the resuspended cells from the third to the thirteenth digestion periods were combined, centrifuged at 90 ***g*** for 7·5 minutes, and resuspended in fresh medium containing 10 per cent foetal calf serum. The cells were counted and diluted to contain $16{\cdot}8–28{\cdot}5 \times 10^6$ cells in the 7-ml starting sample suspensions to be layered over the gradients. The suspended cells that were obtained during the first two 20-minute digestion periods contained many red blood cells and cells which were incapable of excluding Trypan Blue; these were discarded.

Density gradients

The gradient generator and gradient tapping cap which were employed have been described and illustrated in a recent paper (Pretlow *et al.*, 1974b); the tapping cap was slightly modified from that used previously (Pretlow and Boone, 1969). In all gradients, the Ficoll concentration varied as a linear function of the distance from the centre of revolution. Both isokinetic and isopycnic density gradients were collected in 4-ml fractions except for the first fraction which consisted of the 7-ml starting sample suspension. All gradient fractions were treated identically. Refractive indices were measured on all gradient fractions in order to confirm the linearity of the gradients. Cell counts were performed using haemocytometer chambers. Slides were prepared using the Cytocentrifuge (Shandon Southern Instruments, Sewickley, Pennsylvania); duplicate slides from each gradient fraction were stained with Wright stain and Methyl Green–Pyronin as modified by Ahlqvist and Andersson (1972). Differential cell counts were performed counting 200 cells from each gradient fraction and 500 cells from the purest gradient fractions.

Isopycnic centrifugation was carried out using gradients which varied linearly from 4·1 per cent (w/w) Ficoll at the sample–gradient interface, 12·2 cm from the centre of revolution to 43·0 per cent (w/w) Ficoll at the gradient–cushion interface, 23·3 cm from the centre of revolution. Isopycnic centrifugation was carried out using a centrifugal force of 800 ***g*** (measured at the sample–gradient interface) for 90 minutes at 4°.

Velocity cell separation experiments were performed using the previously described isokinetic gradient (Pretlow, 1971) which varies linearly from 2·7 per cent (w/w) Ficoll at the sample–gradient interface, 13·7 cm from the centre of revolution, to 5·5 per cent

(w/w) Ficoll at the gradient–cushion interface 26·7 cm from the centre of revolution. As described previously (Pretlow, 1971; Pretlow *et al.*, 1974b), cells sediment with constant velocities in the isokinetic gradient. At any given centrifugal force, the distance sedimented by any particular cell will be directly proportional both to the duration of centrifugation and to the centrifugal force employed. Since the initial goal of this work was to separate lymphocytes from the other cells in the tonsil, we initially employed identical conditions to those which we had used previously (Pretlow *et al.*, 1973) in the purification of individual kinds of cells from Hodgkin's disease. Accordingly, we used 97 ***g*** for 16 minutes at 4°. As explained later in this paper, in an effort to obtain increased purity, the duration of centrifugation was increased to 20 minutes in some experiments.

RESULTS

SAMPLE COMPOSITION

As mentioned above, dissociation of human tonsils using a variety of physical methods consistently yielded a small number of cells a high proportion of which were unable to exclude Trypan Blue. Digestion of the tonsil with pronase gave a larger number ($16{\cdot}5 \pm 22{\cdot}2 \times 10^6$ cells per gram of which >99 per cent excluded Trypan Blue) of cells which could exclude Trypan Blue than physical methods, but many fewer cells than digestion of the tissue with collagenase or trypsin. While approximately the same numbers of cells were obtained using 0·05 per cent collagenase ($220 \pm 62{\cdot}8 \times 10^6$ cells per gram) or 0·25 per cent trypsin ($226{\cdot}4 \pm 54{\cdot}8 \times 10^6$ cells per gram), the frequency distributions of individual types of cells were different. In five experiments using tonsils from five different patients, digestion with trypsin gave an average of $73{\cdot}5 \pm 1{\cdot}9$ per cent lymphocytes and $4{\cdot}3 \pm 2{\cdot}5$ per cent plasma cells; digestion with collagenase gave $84{\cdot}7 \pm 7{\cdot}6$ per cent lymphocytes and $0{\cdot}3 \pm 0{\cdot}2$ per cent plasma cells. It would appear that collagenase selectively destroys a large proportion of the plasma cells from the human tonsil. In the third to the thirteenth 20-minute digestions, 92–99 per cent of the dissociated cells excluded Trypan Blue when either trypsin or collagenase was used. In the individual experiments in which trypsin and collagenase were used in parallel, the proportions of cells that excluded Trypan Blue were consistently very similar. Because a slightly larger number of cells was obtained using trypsin and because the use of trypsin resulted in a higher proportion of plasma cells, we used suspensions of tonsil cells disaggregated with trypsin for all of the cell separation experiments reported here. The starting sample suspensions of cells which were layered over the density gradients contained $73{\cdot}5 \pm 1{\cdot}9$ per cent lymphocytes, $14{\cdot}0 \pm 1{\cdot}6$ per cent blasts, $4{\cdot}3 \pm 2{\cdot}5$ per cent plasma cells, $5{\cdot}4 \pm 1{\cdot}4$ per cent histiocytes, $0{\cdot}4 \pm 0{\cdot}5$ per cent granulocytes, and $2{\cdot}5 \pm 0{\cdot}4$ per cent red blood cells.

ISOPYCNIC SEPARATION

Isopycnic cell sedimentation resulted in a slight degree of purification of some cell types; however, since the degree of purification was always much less than that obtained using velocity sedimentation, and since velocity sedimentation does not require that the cells be exposed to the high centrifugal forces used in isopycnic centrifugation, only three isopycnic cell sedimentation experiments were performed. In two additional experiments, we attempted to gain plasma cells with an increased degree of purity by centrifuging the separated plasma cells from the plasma cell zone of the isokinetic gradient in a second

step using isopycnic centrifugation. While analogous two-step procedures for cell separation, first according to sedimentation velocity and then according to differences in the densities of cells, have proven useful in previous cell separation problems (Pretlow and Cassady, 1970; Pretlow, Pichichero and Hyams, 1971), the second step (isopycnic centrifugation) did not enhance the purity of plasma cells separated from human tonsils.

SEPARATION IN THE ISOKINETIC GRADIENT

Following sedimentation in the isokinetic gradient for 16 minutes using a centrifugal force of 97 *g* at 4°, the modal population of lymphocytes was located in fraction 9 (±1 fraction) from the isokinetic gradient (Fig. 1); lymphocytes were 93·6±1·9 per cent of the

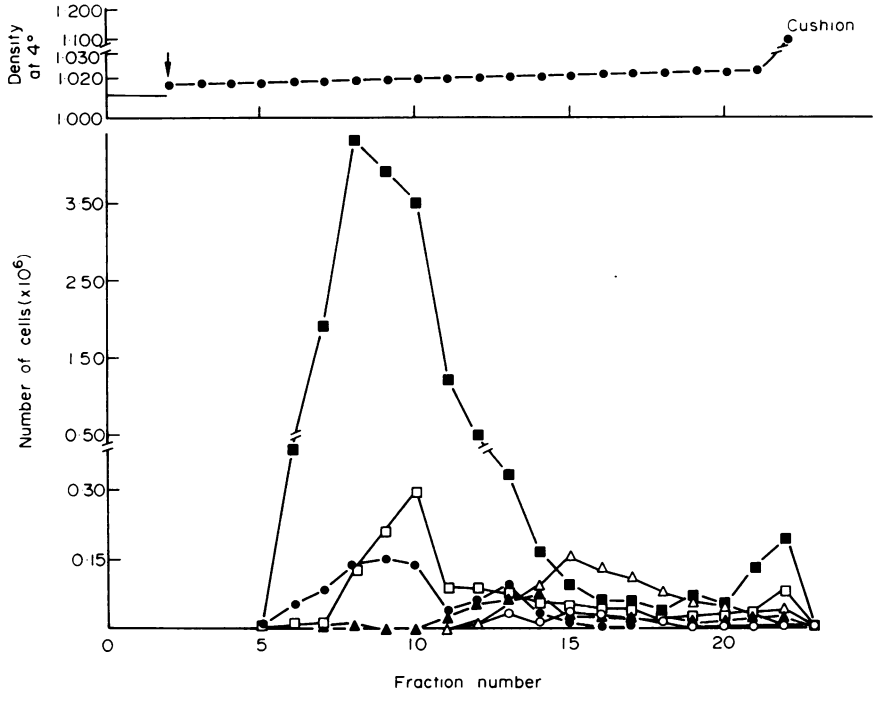

FIG. 1. Separation of cells from the human palatine tonsil using the isokinetic gradient of Ficoll in tissue culture medium. Centrifugation is carried out at 97 *g* (measured at the sample–gradient interface 13·7 cm from the centre of revolution) for 16 minutes at 4°. An arrow marks the sample–gradient interface on the density plot. (■) Lymphocytes. (□) Blasts. (△) Plasma cells. (▲) Histiocytes. (○) Neutrophils. (●) Red blood cells.

nucleated cells in this fraction. The purest lymphocytes were located in fraction 8 (±1 fraction); in this fraction, lymphocytes were 97·2±1·9 per cent of nucleated cells (Table 1). The modal population of plasma cells was located in fraction 15 (±1 fraction) and contained 25·2±12·6 per cent plasma cells. The purest plasma cells were located in fraction 18 (±1 fraction); in this fraction, plasma cells were 33·9±13·9 per cent of all cells and 34·5± 13·7 per cent of nucleated cells.

As is illustrated in Fig. 1, after centrifugation for 16 minutes at 97 *g*, the modal population of plasma cells was just emerging from the more rapidly sedimenting lymphocytes in

TABLE 1

SEPARATION OF LYMPHOCYTES AND PLASMA CELLS BY VELOCITY (ISOKINETIC) SEDIMENTATION*

	Lymphocytes	Plasma cells
Cell suspension before separation	73·5 ± 1·9 (75·4 ± 1·8)	4·3 ± 2·5 (4·4 ± 2·5)
Sedimentation at 97 *g* for 16 minutes		
Modal fraction number	9 ± 1	15 ± 1
Purity of modal population	91·6 ± 1·9 (93·6 ± 1·9)	25·2 ± 12·6 (25·9 ± 11·8)
Purest fraction number	7 ± 1	18 ± 1
Purity after separation	94·4 ± 1·9 (97·2 ± 1·9)	33·9 ± 13·9 (34·5 ± 13·7)
Sedimentation at 97 *g* for 20 minutes		
Modal fraction number	11 ± 1	19 ± 1
Purity of modal population	92·9 ± 0·8 (94·9 ± 0·8)	36·7 ± 1·5 (36·7 ± 1·5)
Purest fraction number	8 ± 1	19 ± 1
Purity after separation	95·3 ± 1·6 (97·2 ± 1·9)	43·1 ± 5·9 (43·1 ± 5·9)

* Purity is expressed as mean percentage of cells ± standard deviation for all experiments. In parentheses, purity is expressed as mean percentage of nucleated cells ± standard deviation for all experiments. The purest plasma cells (43·1 ± 5·9 per cent plasma cells) were obtained after centrifugation for 20 minutes.

the leading edge of the modal population of lymphocytes. In an attempt to resolve these two populations of cells more completely, we decided to sediment the modal population of plasma cells through the isokinetic gradient as far as possible while keeping them out of the aggregates of heterogeneous types of cells at the gradient–cushion interface. In examining the separation shown in Fig. 1, it appeared that the above stated objective could be accomplished by moving the modal population of plasma cells to fraction 19, i.e. the plasma cells would be sedimented approximately 25 per cent further through the gradient in order to reach fraction 19. Since the gradient is isokinetic, this goal was accomplished (Fig. 2) by sedimenting with the same centrifugal force (97 *g*) while prolonging the duration of centrifugation by a factor of 25 per cent (4 additional minutes). After centrifuging with a centrifugal force of 97 *g* for 20 minutes, the modal population of plasma cells was located in fraction 19 (± 1 fraction) and contained 35·7 ± 1·5 per cent plasma cells. While the purest fraction of plasma cells was often adjacent to, rather than identical with, the fraction which contained the modal population of plasma cells, the purest fraction was also fraction 19 (± 1 fraction) and contained 43·1 ± 5·9 per cent plasma cells. The range of purity observed in the purest fraction of plasma cells from the tonsils of different patients was 37·0–48·8 per cent plasma cells. In the gradient fractions that contained the purified lymphocytes (Fig. 3) and plasma cells (Fig. 4), more than 95 per cent of cells excluded Trypan Blue.

RECOVERY OF CELLS

Following velocity sedimentation for 16 minutes using a centrifugal force of 97 *g*, 69·7 ± 13·5 per cent of the lymphocytes and 51·8 ± 23·4 per cent of the plasma cells originally layered over the gradients were recovered in the fractions from the gradients. Following velocity sedimentation for 20 minutes, 68·2 ± 18·8 per cent of the lymphocytes and 38·1 ± 14·4 per cent of the plasma cells originally layered over the gradients were recovered in the fractions from the gradients. Some of the attrition of cells was probably caused by the wall effect. The wall effect artifact is observed in all gradient sedimentation which is carried out in cylinderical, parallel-walled centrifuge tubes and has been reviewed recently (Pretlow *et al.*, 1974b).

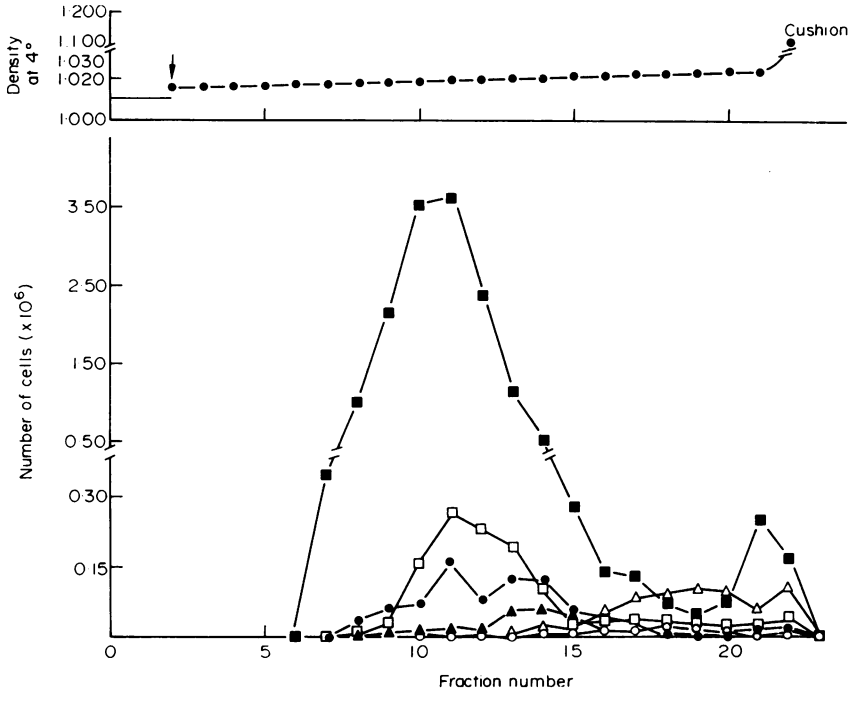

FIG. 2. Separation of cells from the human palatine tonsil under conditions identical to those used for the separation which is shown in Fig. 1, except that centrifugation was carried out at 97 *g* for 20 minutes. The modal population of plasma cells is more widely separated from the modal population of lymphocytes when centrifugation is carried out for the longer period of time (20 minutes). The 20-minute centrifugation also results in a slightly more complete purification of plasma cells as judged from the mean purity obtained in five experiments which employed five different patients (Table 1). An arrow marks the sample–gradient interface on the density plot.

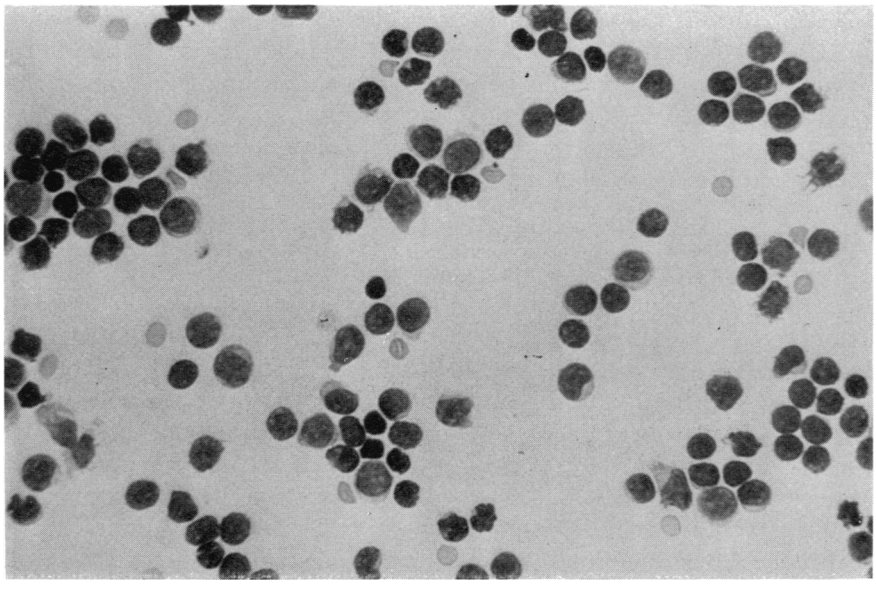

FIG. 3. Cells from fraction 11 of the isokinetic gradient following separation of cells using 97 *g* for 20 minutes. In five experiments with five different patients, 92·4 ± 0·8 per cent of cells and 94·9 ± 0·9 per cent of nucleated cells in fraction 11 were lymphocytes. (Wright stain, magnification × 175.)

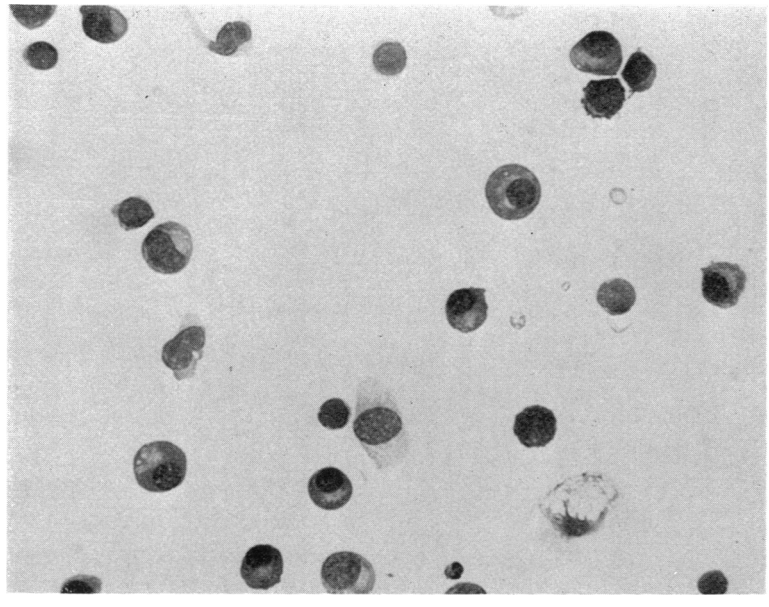

FIG. 4. Cells from fraction 19 of the isokinetic gradient following separation of cells using 97 ***g*** for 20 minutes. In five experiments with five different patients, 43·1 ± 5·9 per cent of cells in fraction 19 were plasma cells. (Wright stain, magnification × 175.)

DISCUSSION

In this paper, we have compared various methods for the dissociation of human tonsils into single cells. As reviewed recently (Pretlow *et al.*, 1974b), the selection of an optimal method for the disaggregation of a particular tissue is highly dependent upon the tissue to be dissociated and the species from which the tissue is obtained. Even in the case of commonly studied laboratory mice, it is increasingly evident that our knowledge of lymphoid cell function is very dependent upon the methods which have been employed to prepare the lymphoid cells. Steinman and Cohn (1973) recently discovered a new type of cell from mouse lymphoid tissues. They emphasized the fact that the frequency distributions of the cell types which they obtained were highly dependent upon the techniques which were employed for the dissociation of lymphoid tissues. In their discussion of the methods which they employed, they found that the conventional mechanical disruption was very limiting and that 'collagenase treatment is much preferred over manual dissection alone' (Steinman and Cohn, 1973). We are not familiar with any previous studies of methods for the dissociation of the human tonsil; however, in the work reported here, we found that more plasma cells were obtained using trypsin than were obtained using collagenase, pronase, or purely mechanical techniques for tissue disruption.

The lymphocytes which we have separated from tonsils are less pure than lymphocytes which we have purified previously from human peripheral blood (Pretlow and Luberoff, 1973) and from the tumour of Hodgkin's disease (Pretlow *et al.*, 1973). In purifying lymphocytes from transplantable rodent tumors, we found that an initial passage of the tumour cell suspension through columns of glass beads before using the isokinetic gradient greatly enhanced the purity of the purified lymphocytes (Zettergren *et al.*, 1973). A similar

purification step could be employed in the purification of lymphocytes or plasma cells from the human tonsil; however, one should be aware of the increasing evidence that columns of glass beads select, at least under certain experimental conditions (Hunt, 1973), not just for lymphocytes, but for specific subpopulations of lymphocytes. Recent successes in the purification of individual kinds of lymphoid cells suggest that electrophoresis may be another useful modality to be used as a second step in the purification of subpopulations of lymphocytes and plasma cells (Hannig and Zeiller, 1969; Häyry, Andersson and Nordling, 1973; Sabolović and Dumont, 1973; Zeiller, Pascher and Hannig, 1970; Zeiller, Holzberg, Pascher and Hannig, 1972). Shortman has recently reviewed other cell separation procedures which might provide useful additional, complementary steps for the purification of tonsillar lymphocytes and plasma cells.

During the past few years, there have been several techniques for cell separation which have been applied to the analysis and separation of antibody-forming cells; these techniques have been reviewed in detail recently (Pretlow *et al.*, 1974b). In most cases, there has been no attempt to differentiate between plasma cells and the other kinds of lymphoid cells (Buxbaum, 1973; Miller, Avrameas and Ternynck, 1973; Saito, Uda, Mori, Miyamoto, Takenaka and Nagai, 1973; Furth, Schuit and Hijmans, 1966) which have demonstrated the capacity to form antibodies. In many cases, there has been no attempt to assess the purity of the separated cells even with regard to the attribute being used for the detection of these separated cells, i.e. ability to form haemolytic plaques, ability to fix specific antigen, etc. If plasma cells are to be functionally characterized and clearly differentiated from other kinds of cells which have the capacity to form antibodies, their characterization will be facilitated by the availability of techniques for the separation of plasma cells from the other cells which dilute them in lymphoid tissues. In our experience, the human tonsil that has been disaggregated with trypsin provides the cell suspension that contains the highest concentration of benign human plasma cells. The technique for separation reported here gives the purest preparation of human plasma cells which has been reported.

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