



A SYSTEM TO MAXIMIZE THE *IN VITRO* IMMUNOLOGICAL STABILITY OF HUMAN LANGERHANS CELLS

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Abstract—Langerhans cells (LC) are now known to be immunocompetent antigen-presenting cells (APC) that are involved with numerous immune reactions initiated in skin, including the induction of contact hypersensitivity and the rejection of skin grafts. Immortalized cells that closely resemble *in vivo* LC have not been developed and mature LC, *in vitro*, appear to only briefly pass through a stage of differentiation when immunological potential seems stable. The literature supports the concept that mature LC possess numerous ultrastructural and antigen (Ag) characteristics. These include surface ATPase activity, Fc receptors, MHC class II Ag, CD1a Ag and Birbeck granules. These studies were undertaken to determine the ideal conditions of LC isolation and enrichment for persistence of these characteristics, such that toxicological and immunoreactive studies could closely mimic the *in vivo* situation. Studies were carried out to evaluate sources of tissue, duration of manipulations, chemical perturbation, stored *v.* fresh tissue, methods of enrichment, donor serological information and cost. LC were evaluated for ultrastructural and Ag stability by light and electron microscopy utilizing monoclonal antibodies (Mab) to surface Ag and ultrastructural features. It was determined that the most critical conditions included duration of manipulations and method of enrichment. Differences between fresh-frozen and fresh tissue samples were negligible. Cells evaluated less than 18 hr post-collection and enriched by density gradient centrifugation consistently demonstrated stability while ATPase activity, MHC Ag and ultrastructural characteristics varied when other methods were used. Implications for immunological and toxicological testing of *in vitro* skin samples can be drawn.

INTRODUCTION

Since the initial light microscopic observations by Langerhans (1868) of epidermal Langerhans cells (LC), these poorly understood skin components have interested researchers. LC are now known to be immunocompetent antigen-presenting cells that are intimately involved with numerous immune reactions that are initiated in skin and have been called the peripheral outpost of the immune system (Parent *et al.*, 1989; Romani *et al.*, 1989; Stingl *et al.*, 1978). Tests of function conducted *in vitro* also show LC to be required for epidermal cell (EC)-induced syngeneic and allogeneic T-cell activation and for EC-generated cytotoxic T-cell responses. *In vivo* they appear to be responsible for the induction of contact hypersensitivity to haptens and seem to take part in skin graft rejection and in the presentation of bacterial, viral and tumour antigens to T lymphocytes. Mature LC possess numerous ultrastructural and antigenic characteristics. These include Birbeck granules (Birbeck *et al.*, 1961), surface ATPase activity (Wolff and Winkelman, 1967), Fc receptors (Schmitt *et al.*, 1990), class II antigens (Romani and Schuler, 1989) and a CD1a antigen (Ruco *et al.*, 1989). Immortalized

cells that closely resemble LC *in vivo* have not been developed, and mature LC *in vitro* appear only briefly to pass through a stage of differentiation when their immunological potential seems stable.

The present studies were undertaken to determine the ideal conditions for LC isolation and enrichment and the persistence of ultrastructural characteristics and antigenic markers such that toxicological and immunoreactive studies could closely mimic the *in vivo* situation.

MATERIALS AND METHODS

Three sources of tissue were evaluated: (1) skin from donors collected using a suction blister device; (2) skin obtained from surgical pathology in the University of Maryland Hospital; and (3) the Transplant Resource Center of Maryland. From the last of these sources medical background and serological information were available at the time of tissue procurement. Irrespective of the source, all tissue was transported to the laboratory in RPMI 1640 plus antimicrobials but without serum. The tissue was either processed immediately or placed in freezer medium [10% glycerol and minimal essential medium (MEM), Sigma Chemical Co.] and stored at -80°C at a rate of freezing of $-1^{\circ}\text{C}/\text{min}$. Epidermal cell suspensions (ECS) were prepared from the sources

Abbreviations: EC = epidermal cell; ECS = epidermal cell suspensions; LC = Langerhans cells; MEM = minimal essential medium; SRBC = sheep red blood cells.

above by trypsin treatment of the skin samples followed by separation of the epidermis from the dermis with fine forceps and finally incubation of the epidermis in EDTA buffer followed by gentle teasing in MEM. After filtration through a double layer of gauze, the separated and suspended cells were washed in Hanks' balanced salt solution and then treated with DNAase and after further washing, the cells were counted and viability determined by trypan blue exclusion.

Enrichment of the LC from the ECS was attempted using two techniques (Schmitt *et al.*, 1989). The first was solid-phase immunopanning, which involved the coating of petri dishes with sheep red blood cells (SRBC) tagged with haemolysin. The LC have Fc receptors that bind to the Fc region of the rabbit antibody bound to the attached SRBC. The second technique involved the use of a Ficoll gradient, where epidermal cells were layered onto a Ficoll gradient and density gradient separation was achieved. Enriched and *in situ* LC were characterized by ATPase staining and the presence of CD1a and HLA-DR antigens determined using a peroxidase anti-peroxidase or fluorescent antibody technique with specific monoclonal antibodies, as well as by immunoelectron microscopy. Ultrastructural studies were undertaken using several embedding media as well as various ratios of fixative.

RESULTS AND DISCUSSION

The results of this study demonstrate that the quality of skin obtained from volunteers was inadequate. The small skin samples less than 5 cm² per site had a high level of tissue integrity and cell viability. However, these quantities could not produce adequate numbers of LC for immunological testing. The second source was surgical pathology and in this instance the limiting factors for continuous utilization of this tissue were the large variability in the time of procurement and the risk of infection (for example from hepatitis B or C, human immunodeficiency virus). The resultant cell viability from this source was also low, which was attributed to the long duration between donor excision and dermatome collection. The most successful source of tissue was the tissue bank and in this instance fresh and frozen tissues were available in essentially unlimited quantity. This tissue had to be purchased (\$100/100 cm²); however, the advantages of having complete serology on all donors and extremely well controlled procurement procedures proved to be invaluable.

Immunopanning or solid-phase immunoabsorption resulted in 95% viability of isolated LC but only provided 50–70% enrichment. Also the cells enriched by this procedure were exposed to a variety of other (rabbit and sheep) proteins used in the procedure. The LC enrichment technique found to be the best was the density gradient centrifugation protocol. This

technique resulted in as high as 90% enrichment of LC (with an average of 70–80%) while at the same time being the most gentle to the cells and leaving the cells unencumbered with extraneous proteins. Percentage enrichment was evaluated using ATPase activity staining as described. This marker, specific for LC, has traditionally been used to evaluate enrichment as well as cellular enzymatic activity. Additionally, immunofluorescent staining was used for similar evaluation. LC that had been enriched by way of the immunopanning technique were difficult to stain by either method, perhaps because of the presence of erythrocytes. Also, damage to the cells by rubber policeman removal from the dishes was evident when these techniques were attempted.

When *in situ* sections were examined by light microscopy in order to determine the baseline percentage of LC cells in the tissue, 2–6% of the cells stained positive for ATPase. Positive ATPase staining was also observed for cells from cell enrichment suspensions. The results of attempts aimed at determining the presence of ATPase on cells of LC enriched samples by way of the immunopanning technique were inconsistent owing to variations in the number of total cells present on each slide. Enriched cell samples isolated by Ficoll gradient centrifugation consistently demonstrated a higher percentage of cells showing surface ATPase staining. Immunolabelling of LC surface markers with monoclonal antibodies to CD1a and HLA-DR followed by PAP staining for light microscopic observation and immunofluorescence microscopy using fluorescein-labelled anti-mouse immunoglobulin serum resulted in positively stained cells. HLA-DR and ATPase activity could be observed in *in situ* sections; however, labelling of LC surface markers with these techniques was most successful when using the LC cell preparation 12–36 hr after enrichment.

The length of time from cell enrichment to immunolabelling was important in the detection of the markers. The combination of cell viability and time-to-reaction variables indicated that for the detection of most markers, cells that were stained/reacted between 12 and 18 hr after enrichment demonstrated the optimal viability and reactivity. More cells with HLA-DR markers were observed as the time from enrichment increased. The ultrastructural characteristics classically described in LC were seen in transmission electron micrographs of Epon-embedded LC and in *in situ* sections. These features included an irregular cell membrane and a frequently folded nucleus, lack of desmosomes and Birbeck granules. A 2.5–3.0% glutaraldehyde–paraformaldehyde fixative ratio in phosphate buffer using Epon-embedded sections allowed the best detection of ultrastructural morphology. Immunological surface markers, however, were best evaluated using a 0.5–3% glutaraldehyde–paraformaldehyde fixative ratio.

In summary, the shortest time between procurement, cell separation and use in the assays resulted in

the greatest degree of tissue integrity and cell viability. Differences in fresh-frozen and fresh tissue samples were minor, with a slight decrease in cell viability with the former. Ficoll gradient separation yielded a higher enrichment with equal cell viability and a lower presence of protein contaminants in the enriched suspension. Ficoll gradient enriched cell samples showed a higher percentage with ATPase staining. The length of time from cell enrichment to immunolabelling was important in the detection of antigenic markers (12–18 hr after enrichment showing the optimal viability and reactivity).

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