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Development of human connective tissue mast cells from purified blood monocytes

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Summary. Highly purified subfractions of human peripheral blood monocytes, when cultured in the presence of 30% L cell supernatant and 30% horse serum, assumed all the characteristics that define human connective tissue mast cells. After three weeks of culture, 75% of the cells developed metachromasia and granular chloroacetate esterase staining, and their intracellular histamine levels increased from 0.0 to 50.5 ng/10^6 cells. On electron microscopy, the cells developed intracytoplasmic granules with all the features typical for mature and immature mast cells. Cultured cells bound 55 pg 125I-IgE/10^6 cells, while labelling was negligible with cells prior to culture and with heat-denatured 125I-IgE. Fluorescent staining with anti-IgE increased slightly as well, while staining with monoclonal anti-monocyte and anti-HLA-Dr markers decreased. Purified lymphocytes did not assume mast cell characteristics, and lymphokines did not induce or enhance in vitro mast cell development or IgE binding. The data therefore further support the concept that connective tissue mast cells arise from the monocytoïd lineage.

INTRODUCTION

Mast cells are mononuclear resident cells of connective tissue that play a central effector role in certain immunological reactions. The cells are characterized by their specific ultrastructure, their contents of glycosaminoglycans and histamine, and their high affinity surface membrane receptors for IgE. Recently, evidence has emerged that two types of cells fulfill these criteria of mast cells (Bienenstock et al., 1982): one cell is small and contains only few specific cytoplasmic granules and low levels of mediators. It increases at sites of immunological reactions such as the intestinal mucosa during parasitosis (Bienenstock et al., 1982; Ruitenberg, Elgersma & Kruizinga, 1979), and it is therefore termed the mucosal mast cell (MMC). The other cell is larger, contains more numerous specific cytoplasmic granules and mediators, does not depend on the presence of thymic tissue (Mayrhofer & Bazin, 1981) or the stimulation of lymphocyte products (Ginsburg, Ben-Shahar & Ben-David, 1982) for its development, and it is termed the connective tissue mast cell (CTMC).

Until now, the ontogeny of both cell types has remained a matter of controversy. This holds particularly for the MMC. In the case of the CTMC, we have been able to corroborate in vitro findings of more than a decade ago which suggested that mononuclear phagocytes serve as the potential precursors of murine mast cells (Parwaresch et al., 1971). Using an in vitro culture system with horse serum and L cell supernatants as conditioning factors, we could show that purified rat peritoneal mononuclear phagocytes can differentiate into typical CTMC (Czarnetzki, Hannich & Niedorf,
1979; Czarnetzki et al., 1982; Sterry & Czarnetzki, 1982). More recently, we have been able to adapt these culture methods to man and have grown mast cell-like cells from pleural cavity and peripheral blood leucocytes (Krüger, Sterry & Czarnetzki, 1983).

Several crucial issues remained unresolved with these studies: (i) a low percentage of lymphocytes persisted throughout the cultures, and their role as potential precursors or stimulators was unknown; (ii) because of the long period before the appearance of mast cell characteristics, it was unclear whether only a subpopulation of mononuclear phagocytes could differentiate into mast cells; (iii) one extremely important property of mast cells, their ability to avidly bind IgE, had not been studied with the cultured human cells.

In the present investigation, we have used highly purified human monocyte preparations for seeding. Subfractions of these cells, separated on the basis of cell density, were also used, in the hope to define a precursor-enriched subpopulation among the peripheral monocytes. Finally, IgE binding studies were made on cells prior to and at different times of culture by two different methods. The data show that the cultured cells exhibit a much increased ability to bind IgE. A specific CTMC precursor subpopulation could however not be observed among the peripheral monocytes.

MATERIALS AND METHODS

Cells
Human peripheral blood was fractionated, yielding four to five subfractions each of either pure lymphocytes or 95–100% pure monocytes. A specially developed elutriation procedure was employed, based on cell density (Figdor et al., 1982). The human promonocyte line HL 60 (Collins, Gallo & Gallagher, 1977) and the human histiocytic line U 937 (Sundstrom & Nilsson, 1976) were kept in minimal essential medium (MEM) Dulbecco (Seromed, München, FRG), supplemented with 10% FCS under standard tissue culture conditions.

Lymphokines
Ficoll-Hypaque separated peripheral mononuclear cells from a patient with Sézary syndrome were stimulated with 2-5 μg/ml phytohemagglutinin for 2 hr, followed by another 22 hr incubation of the washed cells in MEM Dulbecco and 2% FCS. The supernatants were active towards neutrophils. They also attracted eosinophils during in vitro chemotaxis (Colley, 1973; for method, see Czarnetzki, König & Lichtenstein, 1976), indicating the presence of biologically active lymphokines.

Methods of culture
Cells were seeded at $2 \times 10^7$/ml MEM Dulbecco, supplemented with 30% horse serum, 30% LCS, 3 mM glutamine, 50 μg/ml penicillin and streptomycin, 100 μg/ml of glutamin and 1% non-essential amino acids (Gibco, Glasgow) in multiwell (2 ml) tissue culture plates (Falcon Plastics, Oxnard, CA). Details have been described previously (Czarnetzki et al., 1982; Krüger et al., 1983; Czarnetzki, Krüger & Sterry, 1983). Cells were harvested after incubation at 4° overnight by repeated rinsing with cold MEM Dulbecco, supplemented with 5% foetal calf serum (FCS). In some cultures, lymphokine supernatant (20%) was added instead of or in addition to LCS. The culture medium was renewed by 50% of its volume every 2–3 days.

Labelling of cells
Human myeloma IgE (PS) was generously provided by Dr T. Ishizaka, Baltimore, U.S.A. $^{125}$I-labelling of the IgE and column purification was carried out, as previously described for murine cells (Czarnetzki et al., 1982; Ishizaka et al., 1977). Briefly, $1 \times 10^6$ cells in 300 μl MEM with 10% FCS were incubated with 0-8 ng $^{125}$I-IgE for 3 hr at 4°. In some experiments, excess (10 μg) unlabelled human IgE or IgG was added 10 min before or together with the labelled material. Denatured $^{125}$I-IgE (63°, 2 hr) served as control for non-specific binding. Unbound label was removed by centrifugation of 50 μl of the cells through a 300 μl FCS cushion in conical plastic tubes. The tips with the cell pellet were cut off and counted for amount of radioactivity. Experiments were conducted in quadruplicate, and the background was subtracted from the data shown.

For fluorescent binding studies, the following reagents were used: monoclonal mouse anti-human monocyte (BRL 9496 SA), anti-human HLA-Dr serum (BRL 9408 SA) and FITC-labelled rabbit anti-human IgE and anti-mouse FITC (both from the Central Laboratory of the Netherlands Red Cross, Amsterdam). The anti-monocyte and anti-HLA-Dr sera were incubated on smears of cells for 30 min, room temperature. For IgE binding studies, $1 \times 10^4$ cells/150 μl MEM Dulbecco with 10% FCS were incubated with 14 μg of IgE or IgG for 4 hr, 4°.
Anti-IgE was then added to the cell suspension for another 30 min, 4°.

**Cytochemistry**

Metachromasia of cells was evaluated on cells suspended in a 0-2% alcoholic (60% ethanol) solution of toluidine blue, pH 3-6. Chloroacetate esterase and butyryl esterase staining of cyt centrifuged smears was performed as previously described (Czarnetzki *et al.*, 1982; Czarnetzki *et al.*, 1983).

**Histamine**

Cells were lysed in 0·8% HClO₄, and histamine was extracted and measured on a spectrofluorometer after the method of Shore, Burkhalter & Cohen (1959).

**Electron microscopy**

Cells were fixed for 30 min in 2·5% glutaraldehyde and postfixed in 1% osmiumtetroxide. Ultrathin sections, stained with uranyl acetate and lead citrate, were examined with a Philips EM 301 electron microscope at 80 kV.

### RESULTS

**Cytochemical reactions and histamine contents of cells**

In cultures from cell subfractions of purified monocytes from 12 different donors, the kinetics of cell differentiation were indistinguishable and did not differ from the changes observed previously with unfractionated human mononuclear cells (Czarnetzki *et al.*, 1983). The earliest signs of mast cell differentiation were noted by days 8–10 and were present in 60–80% of cells during the third week of culture. Data on the development of several important mast cell characteristics after 21 days of culture are summarized in Table 1. Metachromasia, granular chloroacetate esterase and histamine contents much increased while the percentage of butyryl esterase positive cells remained unchanged although the intensity of the reaction increased. None of these changes were observed in culture of purified lymphocytes, nor did the addition of lymphokines influence the differentiation of the monocytes under the culture conditions employed.

**Light and electron microscopy of cultured cells**

The cells underwent obvious morphological changes during the three weeks of culture, as observed by light and electron microscopy. They increased almost three-fold in size, the nuclear/cytoplasmic ratio decreased, and the round nucleus often assumed an excentric position. The most prominent changes, however, were found in the cytoplasm. Most cells showed an abundance of electron-dense granules, at times with fingerprint inclusions which is typical for mature mast cells (Fig. 1). Other cells displayed cytoplasmic vacuoles with varying numbers of vesicles and pro-granule-like formations.

**125I-IgE binding studies**

Binding of 125I-IgE to fresh monocytes, fresh lymphocytes and the monocyte cell lines was not increased above baseline, and this held for binding of denatured 125I-IgE to all cell types shown (Table 2). Cultured monocytes were found to bind significant amounts of 125I-IgE, by comparison (Table 2), although it is uncertain whether the amount of bound IgE shown represents saturation of the cells. 125I-IgE binding could be totally inhibited by preincubation of cultured monocytes with excess IgE, but not IgG. On addition of cold IgE together with 125I-IgE, binding was reduced by 29%–53% in different experiments. The amount of radioactivity bound to cultured cells never exceeded 5% of the total radioactivity added.

**Fluorescent labelling studies**

The majority of monocytes were labelled with anti-monocytes and anti-HLA-Dr antisera prior to culture,
but binding significantly decreased during culture (Table 3). In contrast, no binding of anti-IgE was noted on cells prior to culture, and fluorescence was detectable on only 1-2% of the cultured cells. On rewarming of the cell suspension, capping could however be observed on these cells. Cells preincubated with IgG instead of IgE remained constantly negative. The U 937 and HL 60 lines stained negative for all three antibodies, except for a slight fluorescence in <1% of HL 60 cells on exposure to anti-monocyte serum.

Table 2. Binding of normal and heat-denatured $^{125}$I-IgE to various cell types (mean of three experiments)

<table>
<thead>
<tr>
<th>Cells</th>
<th>$^{125}$I-IgE (pg/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>Fresh monocytes</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Fresh lymphocytes</td>
<td>3.7±4.1</td>
</tr>
<tr>
<td>Cultured monocytes, day 16</td>
<td>55.6±22.1</td>
</tr>
<tr>
<td>HL 60</td>
<td>1.1±0.8</td>
</tr>
<tr>
<td>U 937</td>
<td>2.3±1.7</td>
</tr>
</tbody>
</table>

Table 3. Percentage of fresh and cultured monocytes with positive membrane fluorescence

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Exp. day 0</th>
<th>Exp. day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-monocyte</td>
<td>1. 85%</td>
<td>1. 5%</td>
</tr>
<tr>
<td></td>
<td>2. 90%</td>
<td>2. 1%</td>
</tr>
<tr>
<td>Anti-HLA-Dr</td>
<td>1. 82%</td>
<td>1. 1%</td>
</tr>
<tr>
<td></td>
<td>2. 91%</td>
<td>2. 0%</td>
</tr>
<tr>
<td>Anti-IgE</td>
<td>1. 0%</td>
<td>1. 2%</td>
</tr>
<tr>
<td></td>
<td>2. 0%</td>
<td>2. 1%</td>
</tr>
</tbody>
</table>

DISCUSSION
The data presented here support and add new information to the previous observations on in vitro mast cell development in a feeder-layer free culture system (Czarnetzki et al., 1979; Czarnetzki et al., 1982; Sterry & Czarnetzki, 1982; Krüger et al., 1983). They show that CTMC can indeed develop from peripheral human monocytes and that lymphocytes or lymphocyte
products are not necessary to stimulate this differentiation. This confirms earlier in vivo and in vitro observations by other authors as well (Mayrhofer & Bazin, 1981; Ginsburg et al., 1982). A specific subpopulation of monocytes that might serve as mast cell precursors could however not be identified with the cells separated on the basis of density, although this has yielded subfractions with very different functional properties (Figdor et al., 1982).

With the present data, the essential requirements that make the cultured cells true CTMC are also fulfilled: (i) The cells accumulate highly sulphated glycosaminoglycans, as can be deduced from the metachromasia at low pH (Table 1). (ii) Their granules become strongly positive on cytochemical staining for the chloroacetate esterase enzyme which is practically absent in monocytes (Sterry & Czarnetzki, 1982; Czarnetzki et al., 1983) (Table 1). (iii) Intracellular histamine levels increase greatly (Table 1). (iv) The cells exhibit typical morphological features of mature and also of immature mast cells (Galli et al., 1982) (Fig. 1). (v) Cultured cells exceed by far the ability of fresh monocytes to bind $^{125}$I-IgE (Table 2). This binding of IgE is specific, as shown by the inhibition studies with IgE versus IgG. The fluorescent studies seem far less sensitive to pick up IgE binding, but the data tend to support the findings with the radioactively labelled IgE. They stress, in addition, that the cultured cells are still primarily immature cells, since they bind relatively little IgE. This is also apparent from the low histamine levels compared to cells isolated in vivo (Krüger et al., 1983; Schmutzler et al., 1978) and from the immature granules found in many cells. None of the groups working on mast cell development in animal species have however been able to obtain mature cells by in vivo culture methods.

The decrease of monocyte and HLA-Dr markers on the cultured cells (Table 3) is intriguing, but might just be a culture artifact. The same may hold for the low marker binding of the cell lines studied here. Expression of HLA-Dr markers has been shown to depend on lymphocyte stimulation in some systems (Steinman et al., 1980), and lymphocyte products are absent in the culture media used here. On the other hand, rat mast cells have been shown to lack IgA antigens (Dăeron & Voisin, 1979; Mossmann et al., 1979), and one of us (T.V.) has shown the absence of HLA-Dr staining on mast cells in human nasal polyps by double labelling fluorescent staining techniques (unpublished).

In conclusion, the present data are a confirmation of our previous studies which suggested that monocytes serve as precursors of CTMC. The method employed here offers beyond that the possibility to study developmental and functional aspects of CTMC under normal and pathological conditions.

**ACKNOWLEDGMENTS**

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