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Chemotactic selection of *Tetrahymena pyriformis GL* induced with histamine, di-iodotyrosine or insulin

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Abstract

It has been hypothesized that in phylogeny the encounter between potential signalling molecules and the continously changing cell membrane could result in the formation of a ligand specific receptor. This chemical (hormonal) imprinting is then transmitted to the progeny generations. It is, however, very difficult to know whether the selection of cells with receptor-like patterns or amplification of complete receptor-like patterns led to the formation of the receptor-hormone complex. The new technique of 'chemotactic selection' provides a physiological response-guided selection of cells. It also enables the testing of subpopulations with the characteristic selector ligand. We show here that of three chemotactic ligands (histamine, di-iodotyrosine (T_2) and human insulin), insulin and T_2 selected subpopulations express a significantly high chemotactic response. Since the control medium has a selector capacity itself, we introduced a chemotactic selection coefficient (Ch_{sel}) which facilitates the comparison of all groups. Using this factor we found that insulin ($Ch_{sel} = 1.57$), functions as a strong selector and T_2 ($Ch_{sel} = 0.98$), was a weak selector. Morphometric evaluation of the cells showed a good correlation between chemotactic responsiveness and morphometric characteristics of subpopulations selected with insulin and histamine. T_2 data suggest that the long lasting responsiveness is not general, but might be subpopulation specific. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Chemotactic selection; *Tetrahymena*; Signaling; Evolution; Hormones; Hormonal imprinting; Morphometry

1. Introduction

The ability to recognize different molecules and select between them had a significant role in the early evolution of recognition systems in living organisms. Because the detection of chemical signals was essential to both unicellular and multicellular organisms, the development of ligands and their proper receptors represented a molecular interaction-dependent event. According to Lenhoff's theory (Lenhoff, 1974), molecular selection played a key-role in this process. Although the number of candidate molecules (e.g. short and long peptides) was high, most of them were consumed simply as nourishment, and had no special physiological effect on the target cells. Only molecules 'selected' as efficient signal molecules were able to induce metabolic or other pathways (Lenhoff, 1974). The ligand-receptor mechanism is complex, involving physicochemical characteristics (stereo- and electrochemical properties) and structural matching between ligand and receptor. These factors influence selection and the present day ligand-receptor complex interactions in or-

Abbreviations: Ch_{sel}, chemotactic selection; Ins, insulin; His, histamine; T_2, di-iodotyrosine.

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ganisms are thought to be the result of these processes.

Chemotaxis represents one of the earliest activities of recognition by prokaryotic and eukaryotic cells since it is based on the selective recognition and uptake of food molecules. In the early stages of evolution very simple molecules, such as carbohydrates, amino acids and dipeptides were effective inducers or blockers of this process driven by concentration gradients (Grebe and Stock, 1998). These effects are rather complex, as beside increased metabolic activity and speed of swimming, optimal ligand concentrations are required and duration of the excitation process and the adaptation are also essential in the development of short and long term memory (Morimoto et al., 1991). In the course of phylogenetic development, several distinct and more advanced activities were added that have important roles in processes such as inflammation, wound healing and fertilization. As a consequence of this process, the signal molecules (attractants and repellents alike) acting in these special cases were furnished with more significant signal moieties, over their ancestral nourishment character.

When investigating the evolution of signaling and experimenting with chemotactic processes, one of the most frequently applied model cells is the eukaryotic, ciliated protozoan *Tetrahymena pyriformis* (Csaba, 1985). In addition to its advantageous migratory features, several similarities in the recognition system to the higher ranks of phylogeny make this organism popular in molecular and cell biology. Specifically, it has well characterized membrane structure with binding sites/receptors (localized especially on the cilia) for signal molecules (Csaba and Németh, 1980; Csaba, 1985; O'Neill et al., 1988; Zipser et al., 1988; Christopher and Sundermann, 1995), inducible second messenger mechanisms — cAMP (Csaba and Nagy, 1976), cGMP (Kőhidai et al., 1992), Ca^{2+} -calmodulin (Kovács et al., 1989), inositol phospholipids (Kovács and Csaba, 1990a) — and production, storage and secretion of hormone-like molecules (LeRoith et al., 1980, 1982, 1983). Thus *Tetrahymena* may have a complete 'endocrine system' in one cell. Similarities in the chemotactic responses elicited with the neutrophil chemoattractant formyl-Nle-Leu-Phe and its competitor BOC-derivative (Kőhidai et al., 1994b) as well as molecule and concentration dependent effectiveness of several types of signal molecules, peptide hormones (Kőhidai et al., 1994a), cytokines (Kőhidai and Csaba, 1998), lectins (Kőhidai and Csaba, 1996) and volatile oils (Kőhidai et al., 1995) were described on this organism. The fine discriminative capacity in the case of insulin derivatives with slight physicochemical differences (Csaba et al., 1994) or histamine-serotonine (Kőhidai et al., 1994a), or the failure of chemotactic resonse to non-signal molecule protamine sulphate (Kőhidai et al., 1994a), also support *Tetrahymena* as an ideal model for studying signal molecules in a chemotactic system.

Since these mechanisms require a specific ligand-receptor system, ligand-induced sorting of cells might be a good tool for signal molecule research. The first encounter between the signal molecule and the binding site evokes hormonal imprinting (Csaba, 1980, 1985, 1994) and results in altered responsiveness of progenitor cells in further encounters. Earlier experiments determined whether selection or amplification has a role in the effect of imprinting (Csaba, 1980; Csaba et al., 1982), and the joint role seemed to be likely as the method was not sophisticated enough. Here, we attempt to use ligand-specific chemotactic signals as inducers of selection and a new technique — chemotactic selection — which offers the possibility of evaluating the chemotactic responsiveness to identical ligands of selected subpopulations. We tested histamine, di-iodotyrosine and insulin, whose receptorial detector mechanisms were proved to act in *Tetrahymena* by binding assays (Kovács and Csaba, 1990b), and studies of phagocytosis (Csaba, 1993; Kovács et al., 1996), growth (Hegyesi and Csaba, 1997) and chemotaxis (Kőhidai et al., 1994a).

Specifically, we analyzed (1) whether chemotactic selection has the capacity to select subpopulations of cells possessing altered (increased) responsiveness to the identical ligand? and (2) whether chemotactic selection has the potential to distinguish subpopulations with characteristic phenotypic diversity?

2. Materials and methods

2.1. Cells and culturing

Tetrahymena pyriformis GL cells were maintained in axenic cultures containing 1% Tryptone and 0.1% yeast extract (Difco, Michigan, USA).

Cells of logarithmic growth phase (48 h) were assayed. Cell density was 10^4 cell/ml.

2.2. Hormones and buffers

We used insulin (Actrapid MC, Novo, Copenhagen, Denmark), histamine (Reanal, Budapest, Hungary) and di-iodotyrosine (T_2-Fluka, Buchs, Switzerland). The applied solvent was the sterile culture medium described above. NaCl-phosphate buffer (PBS) (0.05 M phosphate buffer containing 0.9% NaCl at pH 7.2) was used.

2.3. Chemotaxis assay and chemotactic selection

2.3.1. Chemotaxis assay

The chemotactic activity of *Tetrahymena* was evaluated with a two-chamber, capillary chemotaxis assay (Leick and Helle, 1983) modified by us (Kőhidai et al., 1995). In this set-up, an eight channel-micropipette served as the inner chamber of the system filled with the test substance, while the outer chamber microtitration plate was filled with the model cells. The incubation time was 20 min. This relatively short time was advantageous for measuring pure, gradient directed chemotactic responses and prevented contamination from the randomly running chemokinetic responder cells. The optimal concentrations of applied test-substances, determined in our previous work (Kőhidai et al., 1994a), were: insulin 10^{-9} M, histamine 10^{-10} M and di-iodotyrosine 10^{-8} M. Fresh culture medium served as the control in concurrent runs. The control samples were evaluated parallel in each case to eliminate the undesirable disturbances elicited by spontaneous mutations. After incubation, the samples of the inner chamber were fixed in 4% formaldehyde diluted in PBS. The number of cells was determined using a Neubauer haemocytometer.

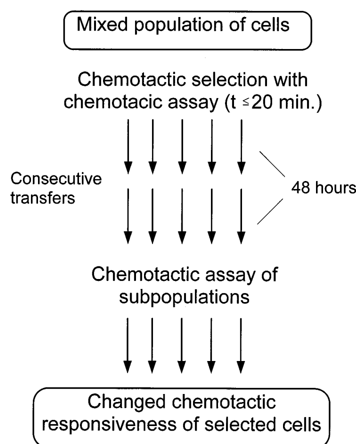

Fig. 1. Schedule of chemotactic selection. Mixed populations of *Tetrahymena* can be selected by chemotaxis to ligands possessing biological signal. Following consecutive transfers of subpopulations started from selected cells the repeated chemotaxis assay demonstrate a changed chemotactic responsiveness of these cultures.

2.3.2. Chemotactic selection

This technique deals with the chemotactic capacity of different signal molecules to form subpopulations of mixed cultures of cells (Fig. 1). First, we applied the chemotaxis assay described above. At the end of incubation, the responder cells were transferred to fresh culture medium for cultivation. Both cultures selected with a known signal substance (S) and controls (C) were consecutively transferred every 48 h. During the 168 h culturing there were three transfers.

Chemotactic responsiveness of cultures was determined again in the following combinations:

S/S-cells selected with the signal substance in the first run and assayed to the signal substance in the second run; S/C-cells selected with the signal substance in the first run and assayed to the control substance in the second run; C/S-cells selected with the control substance in the first run and assayed to the signal substance in the second run; C/C cells selected with the control substance in the first run and assayed to the control substance in the second run.

2.4. Computer-assisted morphometry

Samples were stained with 0.1% toluidine blue for 2 min before analysis. The gently washed cells were placed on microscopic slides and photographed with a light microscope (magnification: 800 ×). The developed prints were scanned with a Hewlett Packard HP-ScanJet IIc scanner. Data were analysed by a computer program (Biomorph 1.1). The interactive morphometry program was able to determine the area, circumference and ratio of the shortest and longest axis (w/l) of the Tetrahymena cells.

2.5. Statistical evaluation of data

All the experiments (chemotactic selection and morphometry) were done in triplicates and the

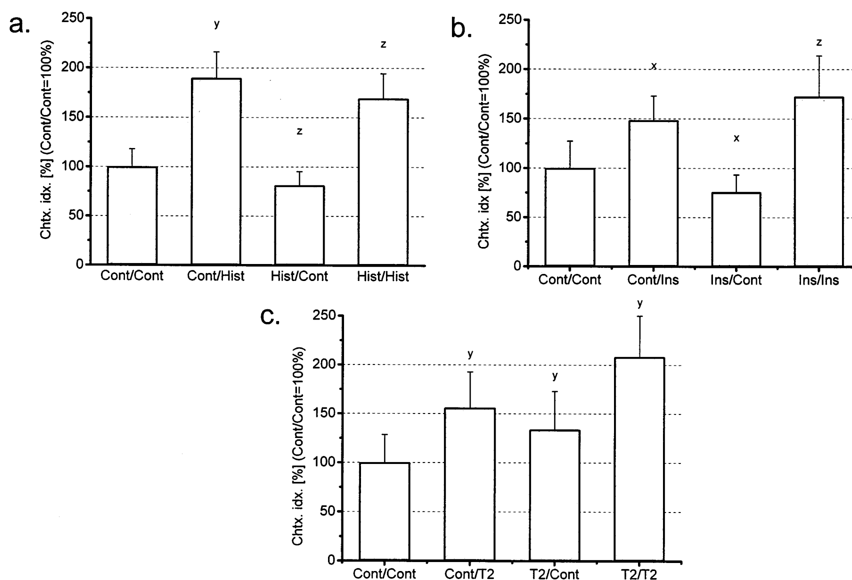

Fig. 2. Chemotactic selection of *Tetrahymena* with (a) 10^{-10} M histamine; (b) 10^{-8} M di-iodotyrosine (T_2); and (c) human 10^{-8} M insulin. (values of y axis are given in % of Cont/Cont; values of significance — $x = P < 0.05$; $y = P < 0.01$; $z = P < 0.001$ — are referred also to the absolute controls). Subpopulations selected with insulin and T_2 express enhanced chemotactic response, since the control substance itself has selector capacity.

experiments were repeated four times. The statistical analysis of data (e.g. variance, standard deviation) were included in the program Biomorph 1.1 and Origin 2.8. For evaluation of significance we applied Student's t-test.

3. Results

3.1. Chemotactic selection

Previous results showed that all three hormones were able to induce positive chemotactic behaviour of the cells (Kőhidai et al., 1994a). These positive responses were also detected in subpopulations selected with the plain culture medium (Fig. 2 a–c; C/Ins $P < 0.01$; C/T_2 $P < 0.01$; C/His $P < 0.05$ columns). We can thus consider these results as relative controls of the successful chemotaxis assay with the identical hormone. However, it was found that the chemotactic potency and activity of the subpopulations selected with chemotaxis was characteristically different.

When cells were selected with the hormones and plain culture medium was applied in the second assay, subpopulations selected with insulin and histamine expressed a significantly decreased chemotactic response. In T_2-selected subpopulations, the adverse chemotactic behaviour was not present, and we noted that these cells had an increased chemotactic response (Fig. 2; Ins/C $P <$ 0.05; His/C $P < 0.001$; T2/C $P < 0.01$ columns).

The second encounter with the 'selector' hormone showed that subpopulations selected with insulin or T_2 showed increased chemotactic responses significantly higher (insulin $P < 0.001$; T2 $P < 0.01$) than responses of the absolute control (C/C) and our relative control groups (C/Ins $P <$ 0.05; and C/T_2 $P < 0.05$). Regarding histamine selection, the second encounter also induced strong chemotactic responses, however they were not significantly different from the relative control group (first encounter).

3.2. Morphometry

Although our model cell possesses a well described oval or pear-like shape, different subsets of cells can be distinguished with morphometry in regard to total area and w/l ratio (ratio of shortest and longest perpendicular axes). In spite of the two similar morphometric indices we should mention that these parameters-area and w/l ratio-have different biological meanings; changes of the size and the shape may be independent.

In the control group, we can distinguish two populations and these findings correlate well with data of the current literature data (Fig. 3). Selection of cells with different hormones influenced this biphasic profile of the control histogram. Selection with T_2 resulted in cultures with four small subpopulations. In contrast, cells selected with histamine resulted in one homogenous cell subpopulation from the mixed culture. In the case of insulin, we were able to detect one pronounced subpopulation with values almost identical to the first peak of the control group. However, in the insulin selected population there is, also, a second, small peak of large cells.

Considering *w*/*l* ratios, we found bi- and triphasic histograms of populations (Fig. 4). In the control group, a biphasic profile was found with dominance of low values-elongated cells and presence of some rounded (postmitotic) cells. These characteristics of the histogram are present in the cultures selected with histamine and insulin. In case of T_2 we could detect, again, a multifocal profile, with dominance of two groups of elongated cells and the presence of the small peak of the rounded cells.

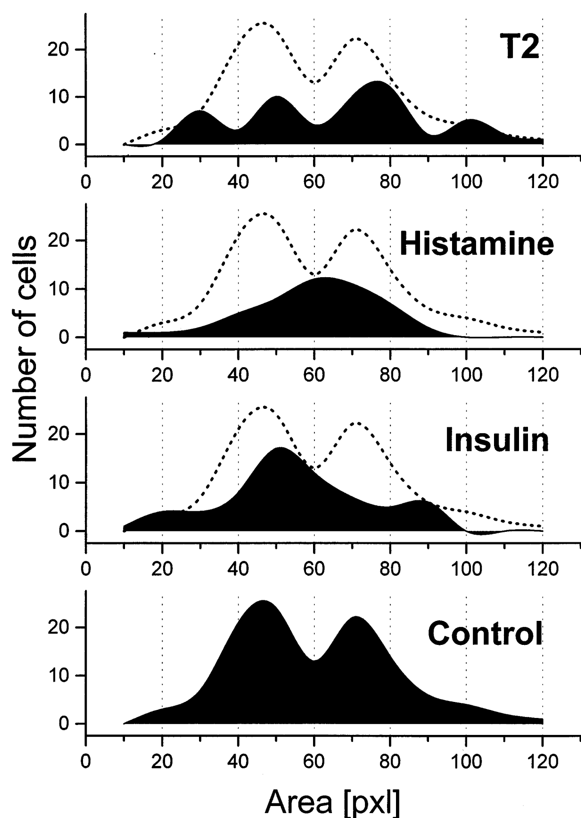

Fig. 3. Computer-assisted morphometry of the area of selected subpopulations of *Tetrahymena*. The single peak histogram of histamine group suggests that histamine has the most narrow range selector ability; the discrete peaks of T_2 histogram point to the presence more, morphologically different subpolulations, despite of chemotactic selection. (S.D. values are not higher than: control, ± 1.91; Insulin, ± 2.42; Histamine, ± 2.34; T_2, ± 3.11).

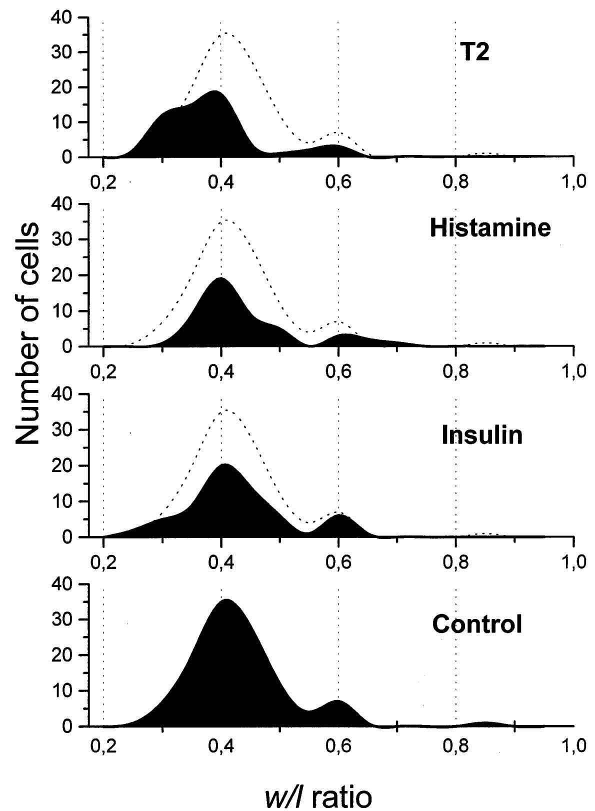

Fig. 4. Computer-assisted morphometry of *w*/*l* ratios of selected subpopulations of *Tetrahymena*. Bi- or triphasic profiles of histograms with dominance of low values are present in the subpopulations selected with control substance, histamine or insulin. The dominance of the first peak in the histogram of T_2 represents postmitotic cells and is characteristic for T_2. (S.D. values are not higher than ± 0.01 in each group).

4. Discussion

As mentioned above, it is not known whether selection or amplification plays the main role in the phylogeny of recognition systems. In hormonal imprinting the first encounter between the continuously changing cell membrane and the molecule suitable for transmitting a signal strengthens the connection. Then if this is advantageous for the cell bearing the 'binding site', the

connection remains constant and the accidental binding site becomes a receptor. This receptor will also be present in the progeny generations, binding the signal molecule, i.e. hormone (Csaba, 1980; Csaba et al., 1982). This could be an explanation of the evolutionary origin of receptor-hormone complexes.

In the present study, our goal was to use chemotaxis — an ancient physiological cell response as a tool in the process of selection and to investigate in the selected groups of cells whether their responses were altered by selection with different ligands. To reduce pretreatment (imprinting) effects, the applied time for the chemotactic run was kept short (20 min) thus allowing mostly chemotactic cell responses. This period was predetermined in our previous work (Kőhidai et al., 1994a). The four combinations in selection of control substance or appropriate ligand at the first and second runs enabled us to evaluate the ligand-specificity of selection and time-dependence of the chemotactic responsiveness in chemotactically selected populations.

In control-medium-selected cells, the control medium itself (composed of tryptone and yeast extract), had a potent selector effect on *Tetrahymena*. Comparing the results of our present study and our previous work-determining different chemotactic responses on *Tetrahymena* with varying concentration courses (Kőhidai et al., 1994a) of the same hormones-shows that the response of cells selected with this control substance was ligand-dependent. The responsiveness increased with T_2 (155 vs. 119%), decreased with insulin (184 vs. 210%) and had no effect on histamine response (148 vs. 148%). A possible explanation for this effect of the control medium is that this reference substance also contains organic components that might have chemotactic effects. Inorganic media, such as Losina-Losinsky solution (Losina-Losinsky, 1931), can be used as they lack organic molecules that might interfere with the chemotaxis. However, they also provide a non-physiological condition for chemotaxis, which is undesirable and therefore fresh, axenic medium was chosen as our control.

Our results showed that histamine, T_2 and insulin have their own chemotactic effect but also have the capacity to select subgroups of cells from mixed cultures, and that the responsiveness of the subgroups is ligand-specific. These hormones have different effects on *Tetrahymena* (Csaba, 1985, 1994), and insulin and histamine are produced by *Tetrahymena* itself (LeRoith et al., 1980; Hegyesi et al., in press). T_2-a precursor of thyroxine -is not a hormone in vertebrates, however in it has a similar and more pronounced effect than thyroxine itself (Csaba, 1994).

The hormones we used represent different types; e.g. size and biochemical characteristics of ligands. Both histamine and T_2 are low molecular weight bioactive molecules with characteristic molecular skeletons. In histamine, the imidazole ring provides characteristic features to the molecule, while the biological activity of T_2 depends on the presence of the aromatic ring and the substitution of two iodine atoms on the ring. Insulin, is a 51-amino-acid-containing polypeptide. It is different from the former two molecules not only in size but in that several sequences of the A and especially of the B chains are prerequisites of biological activity.

Tests with the three ligands showed that the effectiveness of chemotactic selection is molecule-specific and that the chemotactic responses are elicited in these groups both by the control substance (S/C) and the adequate ligand (S/S). This means that selection via chemotaxis provides subpopulations with altered chemotactic responsiveness not only to the selector ligand but on a more general level. This was proved by the comparison of data of insulin and T_2 selected subpopulations. Insulin selection presents a low responder population in general (Ins/C), while T_2 selection has the opposite effect in that the chemotactic behaviour of T_2 selected cells is enhanced to the control (T_2/C) as well. The common effects of T_2 and insulin selection are that both hormones are effective selectors and the chemotactic responses of selected subpopulations (T_2/T_2 or Ins/Ins) was higher than the absolute controls (C/C) and higher than chemotactic activities of these hormones previously observed (C/T_2 or C/Ins). These results show that T_2 and insulin could select cells with adequate chemotactic receptors in the first chemotactic assays. This selection appears also to be long-lasting, since the positive responses to the ligand were detected in the offspring generations after 1 week (170 h) of selection. These long-lasting effects of receptor specific signaling and selection are possibly due to the fact that the selected subpopulations had increased chemotactic activity compared to the subpopulations selected with control medium. On the other hand, the subgroup

of cells selected with histamine recognized the control as absolute and the ligand was also detected as a chemoattractant, but there was not a significantly higher response than in the control selected group.

It is worth noting that the 'main selector' insulin became a recognized hormone during the process of phylogeny, while histamine remained a tissue hormone with local effects. T_2 is considered a precursor of a real hormone.

The response of selected cells to the control substance (S/C) also showed ligand-specificity and points to the receptor mediated character of chemotactic selection. In this respect we can conclude that both insulin and histamine are good selectors as their ligand selected subpopulations expressed low chemotactic response to the control substance, while chemotactic activity of T_2 selected cells was not so specific.

As described above, chemotactic selection and the observed altered responsiveness supports our previous hypothesis that some ligands can characteristically select subsets of cells via chemotaxis. However, differences in the ligand specific selection or responses prompted us to find an evaluation of chemotactic activities which provides a fine balance of the four indexes calculated (C/C, C/S, S/C, S/S). We thus introduced a numerical index that represents the degree of chemotactic selection (Ch_{sel}). This is a ratio where $Ch_{sel} = (S/C \times C/C)/(S/C \times C/S)$. We found that when $Ch_{sel} > 1.25$, these substances promoted a positive response that was very likely receptor mediated and long-lasting. Based on this, insulin ($Ch_{sel} = 1.57 \pm 0.12$) is considered as good selector, possibly via receptor mediated mechanisms. In the case of histamine there was a non-significant ($Ch_{sel} = 1.07 \pm 0.18$), weak selector effect, while the coefficient for T_2 ($Ch_{sel} = 0.98 \pm 0.15$) could not exclude the interaction of receptors. Other mechanisms might also be modifying the chemotactic effects of the subpopulations.

The chemotactic selection study was based not only on the evaluation of chemotactic responses of selected cells. We should consider that there are more subpopulations but they are not essentially chemotactic responders. This relation of chemotactic selection was tested in computer-assisted morphometry assays, which describes well different stages of development or subpopulations of unicellular protozoa. Morphometric evaluation of the area and w/l ratios demonstrated that chemotactic selection has a ligand-dependent potential to form more subpopulations with characteristic phenotypic diversities. In general, the biphasic profiles of area and w/l histograms in control populations shows that the mixed culture itself is not homogenous and contains a small, significant group of cells which might represent a characteristic set of cells with such, still non-characterized physiological properties like chemotactic responsiveness. These basic differences of the profiles of histograms are changed after the chemotactic selections. Based on our data, we can conclude that w/l ratio represents the proper evaluation as comparison of subsets of cells with different areas, nevertheless the more discrete grouping was found in histograms of area (e.g.T2). We can also conclude from the area histograms that the imidazole ring containing histamine has the most narrow range selector ability, resulting in a single peak profile, which shows that this ligand evokes the most uniform subpopulation via chemotaxis. Chemotactic selection with T_2 and insulin evoked more subpopulations. It is worth mentioning that these ligands were effective selectors in respect to chemotactic response at the second encounter as was histamine. T_2 selection resulted in more individual subpopulations (four) than the control or insulin, the best selector according to the coefficient we introduced. However, the dominant peak of insulin is still different from the control profile, which points to the potential of insulin selection to form a morphologically different subpopulation. We do not know, though, whether this morphological separation runs parallel with the enhanced chemotactic response in the offsping, as morphological and functional changes were measured separately.

In conclusion, comparison of the chemotactic activity of subpopulations formed by chemotactic selection and the morphological characteristics of subpopulations support our hypothesis that this form of induced selection can form subsets of cells with both long term chemotactic responsiveness and special morphologic characters. These relations of chemotaxis and morphological properties of cultures raises the issue, whether morphologically different subpopulations possess similar chemotactic responsiveness to a certain ligand or, whether following the selection, there are more, morphologically different and therefore chemotactically 'silent' subpopulations.

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