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Chemotactic selection with insulin, di-iodotyrosine and histamine alters the phagocytotic responsiveness of *Tetrahymena*

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Abstract

Chemotactic selection is a method by which populations of cells exposed to ligands can be isolated and subsequently cultivated. We used *Tetrahymena pyriformis* GL cultures selected by chemotactic selection to insulin (10 nM), histamine (0.1 nM) and di-iodotyrosine (T2, 10 nM) to study the phagocytotic capacity under the induction of selector hormones. Our results show a long-lasting link between chemotactically selected cultures and phagocytotic activity. Cells selected to histamine produced the highest phagocytotic activity upon a second exposure to the selector hormone. T2 selection was also strongly effective, however, the phagocytosis stimulation was not specific to the hormone given later. Insulin selected sub-populations had different phagocytotic responses to the control substance itself, whereas histamine selected sub-populations seem to be heterogeneous in the phagocytotic response to histamine. For insulin, the increased endocytotic or metabolic activity was demonstrated by the lack of non-phagocytotic cells. These experiments call attention to the evolutionary role of selection in the later developing receptor-hormone relationship.

Keywords: Phagocytosis; Chemotactic selection; Insulin; Histamine; *Tetrahymena*

1. Introduction

Phagocytosis is the process by which particulate substances are engulfed by a cell. It is one of the most basic and ancient cell physiological activities and is prominent in carnivorous cells such as *Amoeba proteus* (Prush and Britton, 1987), invertebrate macrophages (Beck et al., 1993) and neutrophils (Franc et al., 1999) and in the eukaryotic protozoan *Tetrahymena pyriformis* (Kovács et al., 1996). Phagocytosis plays an important role in the dynamics of a cell's lifetime with pivotal applications in nutrition, immunological responses and as one of the target reactions of chemotaxis. Understanding the processes involved in phagocytosis can aid in the understanding responses of higher organism's to microbial attack. For instance in chronic granulomatous disease or Chediak-Higashi syndrome both phagocytotic activity

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and chemotaxis are impaired (Gaither et al., 1987; Bellinatipires et al., 1992).

One of the frequently applied model cells in the study of phagocytosis and chemotaxis is the eukaryotic, ciliated protozoan — *Tetrahymena pyriformis*. Its similarities to higher ranks of phylogeny include a well-characterised membrane structure and second messenger systems inducible by various ligands such as hormones like insulin (Kőhidai et al., 1992), histamine (Csaba et al., 1976) and indoleamines (Csaba, 1993). In addition, its rapid reproduction rate enables us to check several generations in a short time. This allows us to follow potential changes in phagocytotic activity and possibly determine the durability of internal endocytotic processes over successive generations.

Previous results have shown that different ligands (e.g. ACTH, insulin) exert different effects on the phagocytotic capabilities and response of *Tetrahymena* (Kőhidai et al., 1995). Specific receptor-ligand interactions are responsible for this phagocytotic activity. For example, insulin has a relatively specific receptor in *Tetrahymena* (Kovács and Csaba, 1990) which becomes even more specific after a previous encounter (Kőhidai et al., 1990). Insulin has a slight negative (or no) effect on phagocytosis (Kőhidai et al., 1995) whereas, in contrast, histamine, a phagocytosis stimulating hormone in mammals (Jancsó, 1955; Kuschinsky et al., 1955; Lison and Smulders 1949; Falus, 1994) enhanced the phagocytotic activity of *Tetrahymena* (Csaba and Lantos, 1975). Other ligands, such as opioids, inhibit phagocytosis via receptor mediated endocytosis (Renaud et al., 1995) and some hormones (e.g. insulin, ACTH) were able to influence the uptake of particles and also the release of particles into the environment (exocytosis). These results support the idea that specific receptor ligand binding is responsible for phagocytotic and exocytotic activity in *Tetrahymena.*

Evaluation of the chemotactic responsiveness and phagocytotic activity of selected sub-populations of cells under different conditions is possible with a new technique introduced in our previous work (Kőhidai and Csaba, 1998). This technique 'Chemotactic Selection' was applied in the present experiments to histamine, T2 and insulin.

The main objectives covered in this study were: (1) do ligands capable of inducing chemotactic selection also have the ability to influence to the phagocytotic activity in selected sub-populations of *Tetrahymena* cultures; and (2) is there any relation between sub-populations selected with different ligands and number of non-responder, phagocytotically inactive ('0-cells') of phagocytosis?

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 in the logarithmic growth phase (48 h) were assayed. Cell density was 10^4 cells/ml.

2.2. Hormones and buffers

The hormones used were porcine mono-component insulin (Actrapid MC, Novo, Copenhagen, Denmark), histamine (Reanal, Budapest, Hungary), di-iodotyrosine (T2-Fluka, Buchs, Switzerland). NaCl-phosphate buffer (PBS, 0.05 M phosphate buffer containing 0.9% NaCl at pH 7.2) and Losina-Losinsky solution containing inorganic salts (LL: 1% NaCl; 0.1% Mg Cl_2; 0.1% $CaCl_2$; 0.1% KCl; and 0.2% $NaHCO_3$) were used as diluents and to wash cells in subsequent steps of the phagocytosis assay.

2.3. Chemotaxis assay and chemotactic selection

2.3.1. Chemotaxis assay

The chemotactic activity of *Tetrahymena* cells was evaluated using a modified two-chamber, capillary chemotaxis assay. An eight-channel-micropipette served as the inner chamber of the system and was filled with the test substance. The outer chamber consisted of a microtitration plate filled with the model cells. The incubation time was 20 min. This relatively short time facilitated the measuring of pure gradient, directed chemotactic responses and prevented the contamination of our samples from randomly running chemokinetic responder cells. The optimal concentrations of applied test substances, determined in previous work of ours were: insulin 10^{-8} M; histamine 10^{-10} M; and di-iodotyrosine (T2) 10^{-8} M. Fresh culture medium served as a control substance in concurrent runs. These control samples were

evaluated in parallel in each case to eliminate the possible undesirable disturbances elicited by spontaneous mutations. After incubation, the samples were fixed in 4% formaldehyde diluted in PBS. The number of cells was determined using a Neubauer haemocytometer.

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). We initially applied the chemotaxis assay described above and at the end of incubation, the control (whole cell population without selection) and the responder cells were transferred to fresh culture medium for cultivation. Cultures were selected with a known signal substance (I = insulin, H = histamine or T = di-iodotyrosine) or with the control medium (C) only at one time. Consecutively, the cultures were transferred to fresh medium every 48 h.

2.4. Assay of phagocytosis

After 1 week, each culture was studied using a phagocytosis assay. Cells were pre-incubated for 3 h in LL solution to have starved model cells with particle free cytoplasm. Volumes of starved cultures, suspension of Chinese ink and agonists were mixed (v/v/v = 1:1:1). They were then fixed with 4% formaldehyde containing LL solution and the test particle number was determined by light microscope in 100 cells per group. In each

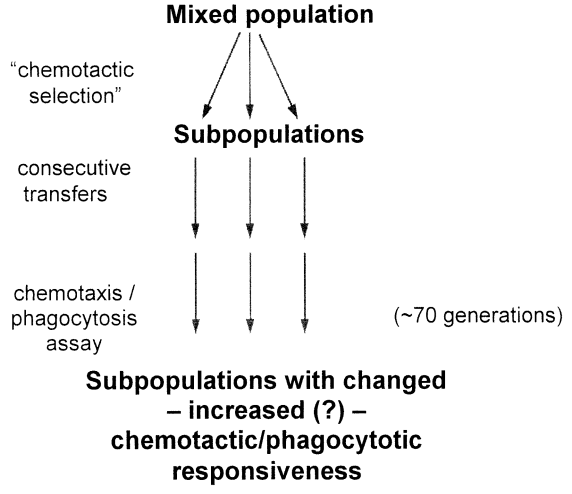

Fig. 1. Review scheme of chemotactic selection technique.

Table 1
Effect of chemotactic selection with hormone ligands on the phagocytic responsiveness of *Tetrehymena* cells on treatment with identical or non-identical ligands. The values are given in percents of the absolute control (C) ±S.D. values are less than 9.53. Results of *t*-test (two-tailed) are shown when differences from the absolute control are significant (*x*, $P < 0.05$ and *y*, $P < 0.01$).

	Treatments				
		C	Insulin 10^{-8} M	Histamine 10^{-10} M	T2 10^{-8} M
Selected by	C	100	108	106	109
	I	109	112	107	115
	H	120	112	137 (*x*)	129 (*y*)
	T2	129 (*x*)	130 (*x*)	124	127 (*y*)

selection, we checked the case with each hormone and also the cross-reactivity with other hormones.

The mean number of Chinese ink particles/cell was determined and is given in Table 1. In addition, the distribution of particle quantity in the cells was also determined (Figs. 2–4). Fig. 5 shows the number of '0 cells' (cells without particles).

2.5. Statistical analysis of data

Each assay was repeated in five independent experiments in three replicas of each. Groups treated with the culture medium or groups selected with culture medium and tested with plain medium again were considered as 'absolute control' groups. For each group/experiment mean values of number of test particles were calculated, data-points of figures were calculated from the mean values of identical groups. Identity of histograms were analysed with Chi-square probe, in the case of significance the *P*-values are given on the figures. Other data were evaluated by using statistical tests (ANOVA and two tailed *t*-test) of Microcal Origin 2.8.

3. Results

Chemotactic selection with the applied hormones had an inducer effect on the phagocytosis and sub-populations with different mean phagocytotic activity were found in different combinations of treatment (Table 1). Lowest levels were found for the first treatments and the highest levels after histamine and T2 selection. The cell-

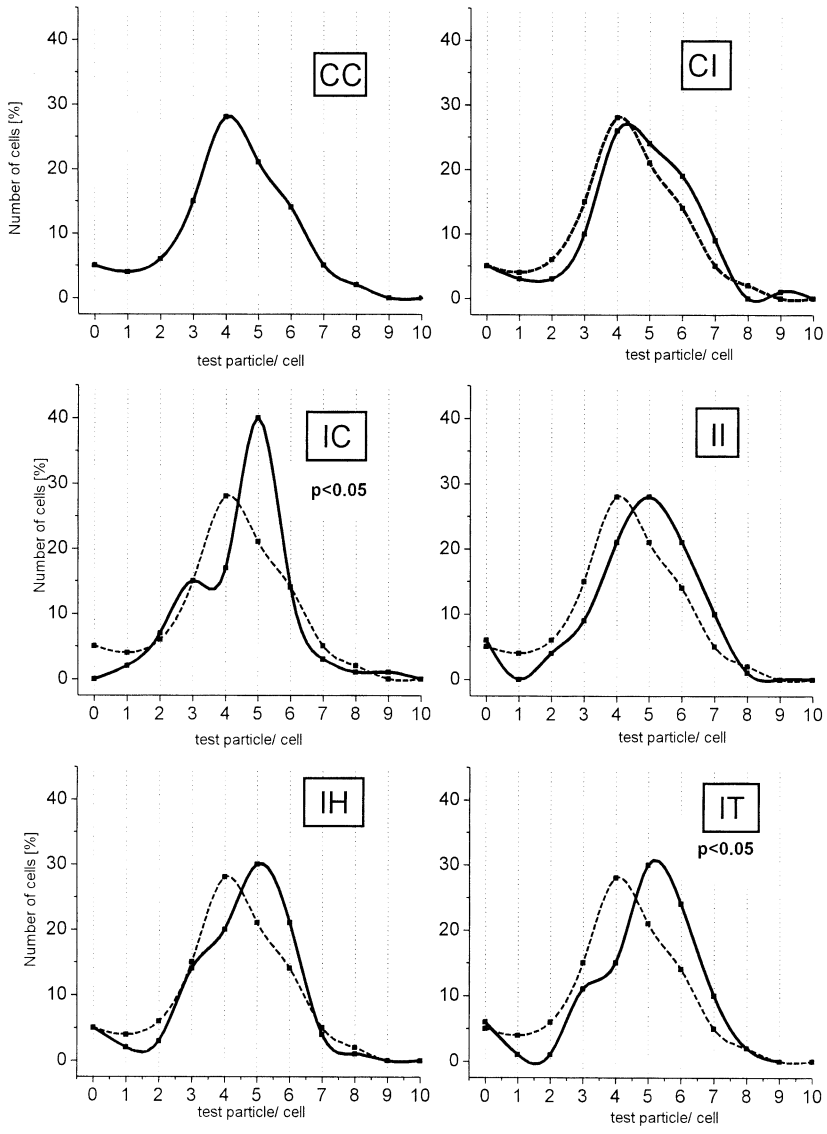

Fig. 2. Phagocytotic activity of *Tetrahymena pyriformis* cultures selected with 10^{-8} M insulin. (CC: control/control; CI: control/insulin; IC: insulin/control; II: insulin/insulin; IH: insulin/histamine; and IT: insulin/T2, dotted line represents the histogram of control) (±S.D. values of data are less than 3.2).

level profiles of the experiments are given in Figs. 2–4.

3.1. Insulin

Tetrahymena cultures selected with control substance expressed no significant change in the phagocytotic activity compared to the absolute control group (CC) when cells were treated with insulin (CI) (Fig. 2). In contrast, phagocytotic responsiveness of cultures selected with insulin was changed, the hormones or control substance could elicit significantly different profiles from the CC group. Comparing CC group to the insulin control (IC) group we obtained a double peak in the insulin curve, which shows that there is a more- and a less-active sub-population of cells concerning phagocytosis. However, when the cells were selected with insulin (II), as opposed to the control (CC), the double peak disappears. In addition, there is also slight right shift of the

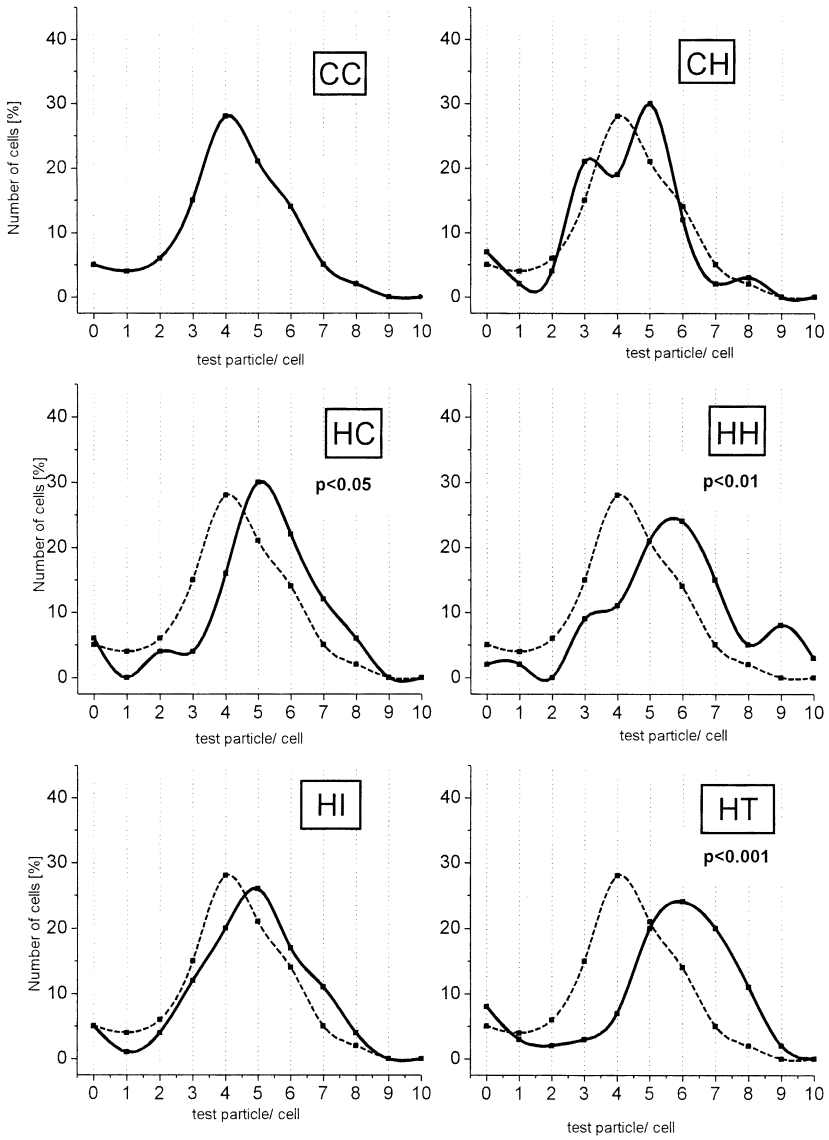

Fig. 3. Phagocytotic activity of *Tetrahymena pyriformis* cultures selected with 10^{-10} M histamine. (CC: control/control; CH: control/histamine; HC: histamine/control; HH: histamine/histamine; HI: histamine/insulin; HT: and histamine/T2, dotted line represents the histogram of control) ($\pm$S.D. values of are less than 3.8).

graph (the number of non-phagocytotic, '0-cells', which are shown later). When insulin selected cells were subsequently exposed to non-identical hormones of the original selection-histamine (IH) or T2 (IT) groups the right shift still appears, albeit with a lower peak than seen in the insulin control run.

3.2. Histamine

We evaluated the altered responsiveness of groups sleeted with control medium and then tested for histamine response (CH). We could again distinguish more peaks/sub-populations (Fig. 3), two of them represent higher phagocytotic activity than the identical control (CC). In the histamine selected cultures, a shift to the right in the control run (HC) was observed, as well as a small subgroup represented by the lower peak in the curve. When these cells were treated with the selector hormone, histamine, (HH) a strong shift to the right was detected and the

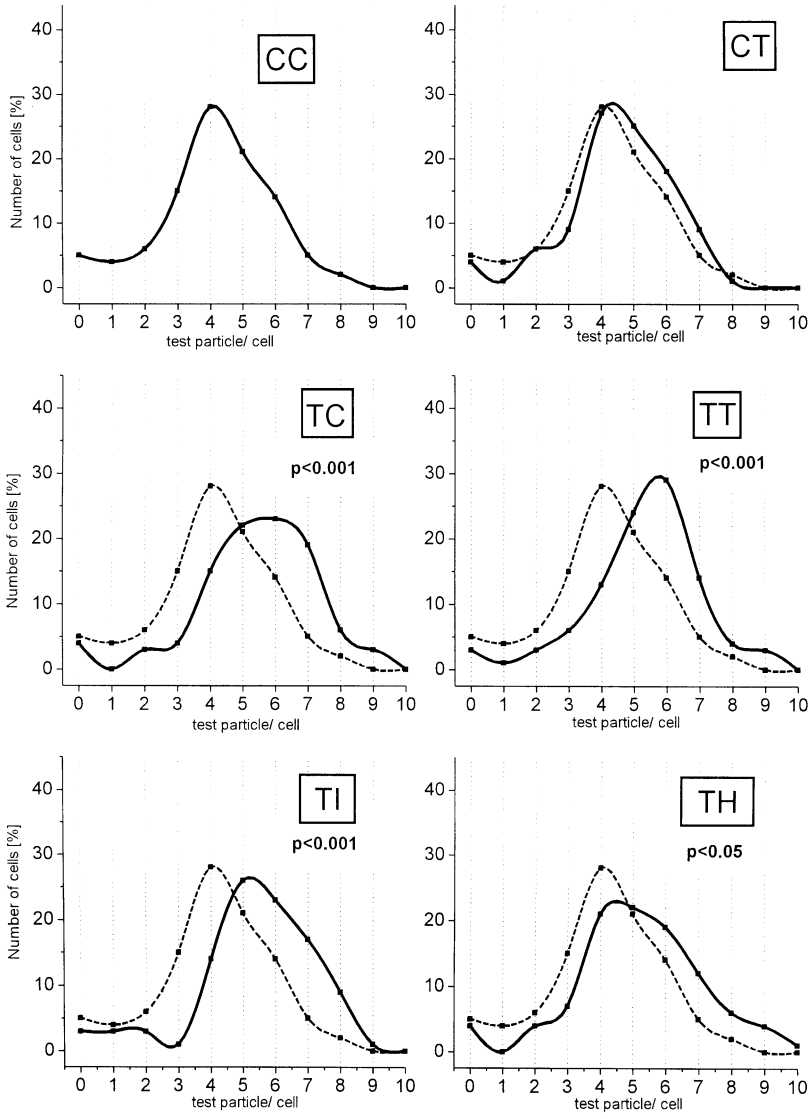

Fig. 4. Phagocytotic activity of *Tetrahymena pyriformis* cultures selected with 10^{-8} M di-iodotyrosine (T2). (CC: control/control; CT: control/T2; TC: T2/control; TT: T2/T2; TI: T2/insulin; TH: and T2/histamine, dotted line represents the histogram of control) ($\pm$S.D. values of are less than 3.5).

three peaks/sub-populations were also distinguishable with a dominance compared to the CH group. This possibly signifies selection of enhanced phagocytotic subgroups by chemotactic selection of histamine. Insulin exerted no effect on phagocytosis when selected cells were with histamine (HI). T2 induced a strong phagocytotic response as demonstrated by the most significant right shift detected in this study, but the pattern followed a more traditional Gaussian curve and the extra peaks (HT) were absent.

3.3. T2

Cultures selected with culture medium did not present altered phagocytotic activity for T2 induction, however, a small sub-population with lower responsiveness is still present (CT) (Fig. 4). Selection with T2 resulted in almost homogenous subpopulations with higher right shifted-phagocytotic responsiveness to the tested identical ligand T2 (TT) or to the non-identical ligands insulin or

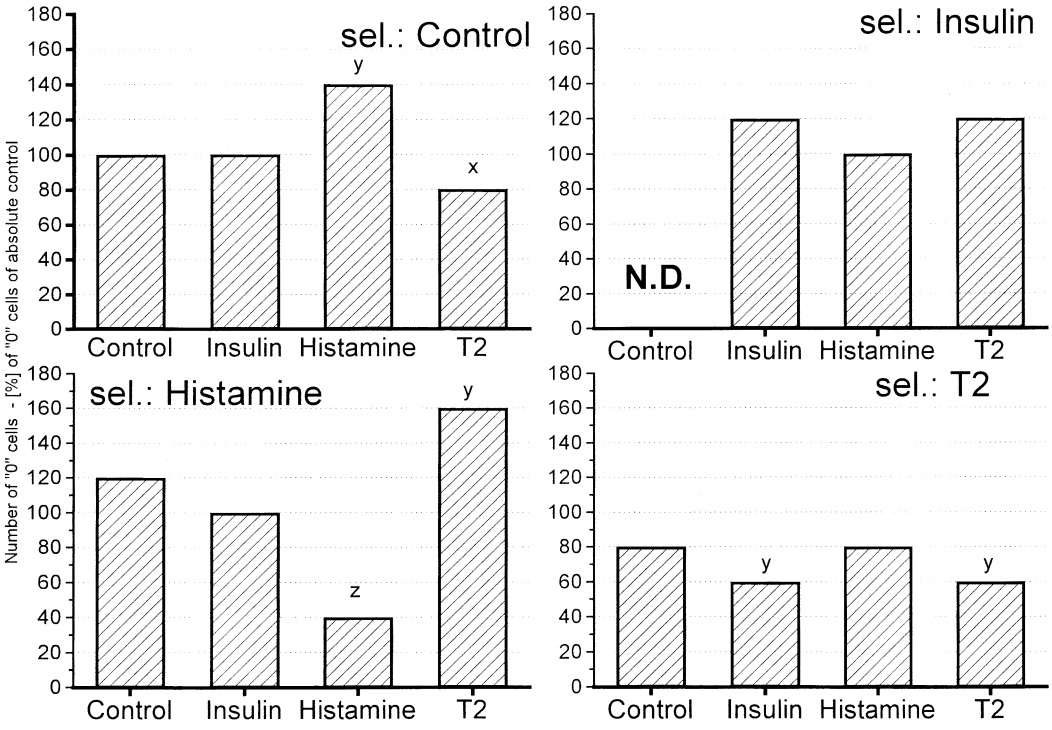

Fig. 5. Number of non-phagocytotic, '0-cells' in chemotactically selected (sel.) *Tetrahymena* cultures treated with 10^{-8} M insulin, 10^{-10} M histamine or 10^{-8} M di-iodotyrosine (T2). (N.D. = '0-cells' were not detectable). Results of ANOVA evaluation are shown in the figure: *x*, $P < 0.05$; *y*, $P < 0.01$; *z*, $P < 0.001$. ($\pm$S.D. values are less than 4.83).

histamine (TI or TH). The phagocytotic activity of T2-selected populations seems not to be ligand-specific, as control medium itself induced a significantly enhanced response (TC).

3.4. Evaluation of phagocytotically inactive, '0-cell' content

Observing the chemotactic selection in the sub-populations of '0' cells, they also provide a guide for evaluating the cross reactivity of hormones acting on the selected cells as the number of '0' cells is indirectly proportional to the phagocytotic induction of hormones. The effect of one hormone can possibly be affected by subsequent selection with another hormone. Selection with the control substance had a significant suppression on the phagocytotic ability in the presence of histamine (increased number of '0' cells) and has a slight stimulatory effect in the case of T2 (decreased number of '0' cells) (Fig. 5a). Insulin selection dramatically stimulates phagocytosis without any concurrent treatment. In addition no cross reactivity was noted with any of the three hormones (Fig. 5b). The selection with histamine caused more uptake of test-particles in the presence of histamine. In contrast, cells treated with T2 had a decreased uptake when selected with histamine (Fig. 5c). The increased phagocytotic activity of T2 selected sub-populations was also portrayed with the number of non-phagocytotic cells. This index was decreased in all treatments applied, however, effect of T2 and insulin was stronger than histamine (Fig. 5d).

4. Discussion

Chemotactic selection has an overall positive effect on the phagocytotic activity of sub-populations of cells responding to identical or non-identical hormones. Histamine can function as a phagocytosis inducer in mammals (Jancsó, 1955; Kuschinsky et al., 1955; Lison and Smulders, 1949; Falus, 1994) and in *Tetrahymena* (Csaba and Lantos, 1973; Kovács and Csaba, 1980) alike. Histamine can elicit its characteristic effect, weak in the case of the control cells and very strong in the

selected cells (Table 1, CH and HH, respectively). This means that cells selected with a single chemotactic selection are most reactive to histamine. In earlier experiments, when the influence of selection and amplification effects was studied (Csaba et al., 1982; Csaba, 1980) the results were uncertain in the absence of suitable methods. Application of chemotactic selection provides a new technique to study the problem. Unselected control and histamine-selected cells have sub-populations with different phagocytotic capacity. However, presence of different sub-populations also calls attention to the fact that chemotactic selection with specific ligands could lead to sub-populations responding differently to either the selector (e.g. histamine, HH) or to the control substance (e.g. insulin, IC). However, this type of selection with a non-specific (control) substance might also be able to present functionally divergent sub-populations (e.g. CH group)

Though selection to histamine resulted in the highest level of phagocytosis (Table 1137%, S.D. = 5.21), selection to T2 also stimulated phagocytosis in very high levels (127% S.D. = 3.82). It was known earlier that T2 increases the number of cell divisions in *Tetrahymena* (Csaba and Németh, 1980). In previous experiments (Csaba and Lantos, 1975), its phagocytosis stimulating effect was similarly low as reported here (Table 1, CT). However, in the T2 selected *Tetrahymena* population this changed and we are uncertain why. There is the possibility that the increase of the metabolic rate under the effect of the hormone is the cause of the phagocytosis stimulation. Nevertheless, the characteristic histamine double peak, (Fig. 3, CH and HH) is not present with T2 (Fig. 4, CT and TT).

In our previous work, we deduced that chemotactic selection has a ligand specific effect on the chemotactic responsiveness of sub-populations of *Tetrahymena* (Kőhidai et al., 2000). Apart from interleukin 8 and TNF-α (Kőhidai and Csaba, 1998), porcine insulin proved to be the most effective selector and induced the highest chemotactic responses.

Comparison of the present data with our previous report on chemotactic selection with similar ligands (Kőhidai et al., 2000) indicates that insulin selects more subgroups with different phagocytotic responsiveness to the selector ligand or foreign ligands and that this type of selection provides more, morphologically distinct, sub-populations. However, their chemotactic responses are still significantly increased in response to insulin. The same comparisons for other ligands show that histamine and T2 have different capacities concerning the phagocytotic ability. Selection with T2 has no ligand-specific effect on the phagocytotic response, however, our morphometry study demonstrated that sub-populations formed by T2 selection are highly heterogeneous compared to the control. For histamine, we conclude the opposite, as morphologically homogenous populations appeared to be phagocytotically distinct from the normal Gaussian curve. All observations suggest that chemotactic selection is a suitable tool to form subgroups with higher responsiveness to the selector ligand, however, these subgroups are not identical with respect to morphology and phagocytosis.

Characterization of phagocytotic samples with the non-phagocytotic, '0 cells' (Kőhidai et al., 1995) was supported theoretically in that this characterisation gives a good index of the inducibility of the phagocytotic mechanisms of cells. By keeping the incubation time short, we were able to eliminate the possibility that these cells were losing their previously phagocytosed tests particles. The dramatically decreased number of '0 cells' in the insulin selected and, subsequently, non-treated group shows that insulin selection has an ability to select group of cells with enhanced endocytotic/metabolic activities.

Data of literature certify the significance of spontaneous or induced selection in phylogeny. Some beneficial mutations of *E. coli* are considered as inducers of punctuated evolution (Elena et al., 1996) analysed by the correlation between average cell size and mean fitness, however, adaptive evolution has some 'speed limits' in asexual populations (de Vesser et al., 1999). While asexual way of growth of our eukaryotic amicronuclear model cell *Tetrahymena pyriformis* GL limits the genetical significance of consequences and data of experiments on flys or mice also demonstrate that response to artificial selection stops when genetic variation is exhausted (Lopez and Lopez-Fanjul, 1993), the main objective of the present experiment was to analyse the signalling mechanisms involved in chemotactic and phagocytotic responses elicited by hormone ligands. The present data support our view that chemotactic selection is a proper tool to characterise receptor-ligand interaction signalling

processes and that phagocytosis, as a target reaction of chemotaxis, is modified in the selected sub-populations. Results gained with *Tetrahymena* demonstrate that the link between chemotaxis and phagocytosis is a closely interactive physiological process with ancient phylogenetical backgrounds.

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