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Comparison of the internalization efficiency of LDL and transferrin receptors on L_2C guinea pig lymphocytes

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We demonstrate that L_2C lymphocytes have about 10-times more receptors for transferrin (Tf) than healthy lymphocytes, as has been shown in the case of LDL receptors. The dissociation constant is the same in the two cell types (about 4×10^{-7} M). In contrast to LDL, Tf enters L_2C lymphocytes with very rapid kinetics. It is shown by cross-reaction that each receptor is internalized independently of the other.

Lymphocyte *LDL* *Receptor-mediated endocytosis* *Transferrin*

1. INTRODUCTION

Many cells internalize macromolecules by receptor-mediated endocytosis. This process involves the binding of a ligand to a specific cell surface receptor, and the subsequent internalization of the receptor-ligand complex by means of clathrin-coated pits and vesicles [1]. Low density lipoproteins (LDL) enter a large number of different cells via a receptor-mediated pathway, and are delivered to lysosomes, where they are degraded [2]. In some malignant cells, the number of LDL receptors on the cell surface is greatly increased [3,4]. We have demonstrated a 10-fold increase in the number of LDL receptors on the surface of L_2C leukemic guinea pig lymphocytes, whereas the corresponding LDL uptake is only twice that of normal lymphocytes [5].

Transferrin (Tf), the major iron-binding protein in plasma, carries this ion to various tissues. Receptors for Tf have been found on the surface of cells requiring iron, such as red blood cell precursors [6] and more recently, transformed cells [7,8]. In the following study, we show that the same leukemic lymphocytes have a large number of Tf receptors compared to normal lymphocytes, but no impairment of Tf internalization has been found. Lastly, we have simultaneously compared the activity of LDL and Tf receptors, and show that the two receptors work independently.

2. MATERIALS AND METHODS

2.1. *Cells*

The L_2C leukemia affecting the cells used in this study arose spontaneously in a strain 2 guinea pig [9] and was passaged in syngeneic animals. L_2C lymphocytes were harvested and purified by Lymphoprep gradient centrifugation, as described [10].

2.2. *Preparation of ^{125}I-LDL and ^{125}I-Tf*

Human LDL (d 1.019–1.063) was isolated from healthy donors, by the method of Havel et al. [11], and radiolabeled (spec. act. about 200 cpm/ng protein) as in [5].

Human Tf (Sigma) was saturated with iron [12] prior to iodination. Radiolabeling of Tf was performed with immobilized Enzymobead lactoperoxidase-glucose oxidase (Bio-Rad). Diferric Tf (400 μg) was combined with 50 μl Enzymobead reagent, 1 mCi $Na^{125}I$ (Amersham) and 50 μl of 2% D-glucose, and kept at room temperature for 30 min. The reaction mixture was then passed through a PD10 column (Pharmacia). The pooled fractions corresponding to ^{125}I-Tf were assayed for

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proteins [13]. The specific activity in the preparation varied from 500–1000 cpm/ng protein.

2.3. *Binding and uptake assays*

Binding and uptake of ^{125}I-LDL were performed as described in [5], with or without an excess of unlabeled Tf. To measure the binding of ^{125}I-Tf, L_2C lymphocytes were incubated at 4°C with various amounts of ^{125}I-Tf in phosphate-buffered saline plus 5 mg/ml bovine serum albumin (PBS-BSA) in a total volume of 0.1 ml. At the end of the incubation period (1 h), 1 ml ice-cold medium was added and the radioactive medium removed by aspiration after centrifugation. The cell pellet was resuspended with 50 μl PBS-BSA and layered over 0.5 ml dibutylphthalate oil/0.5 ml sucrose (15%). After 1 min at 13000 rpm (Beckman Microfuge 11), the tubes were frozen in liquid nitrogen, the bottom of the tubes cut off and cell radioactivity counted. Nonspecific binding was determined in the presence of 260 μg unlabeled Tf. ^{125}I-Tf uptake was carried out at 37°C in a total volume of 0.2 ml. The proportion of internalized ligand was determined as described by Klausner et al. [12].

The LDL dependence of Tf internalization was studied in binding and uptake experiments with ^{125}I-Tf in the presence of an excess of unlabeled LDL.

2.4. *Pulse-chase study of ^{125}I-Tf binding and internalization in L_2C cells*

^{125}I-Tf was allowed to bind to the cells at 4°C as described above. After washing off excess unbound ligand, the cells were incubated at 37°C following addition of prewarmed medium containing 30 μg/ml unlabeled Tf. At the indicated times, the cells were quickly chilled by dipping the tubes 3 times into liquid nitrogen, and transferring them to 4°C. Total uptake and internalized ligand were determined as described above.

3. RESULTS

Binding of ^{125}I-Tf to normal and L_2C guinea pig lymphocytes at 4°C was saturable and time-dependent. Saturation was achieved within 10 min. As shown in fig.1, the amount of ^{125}I-Tf bound to L_2C lymphocytes at 4°C was higher than in the

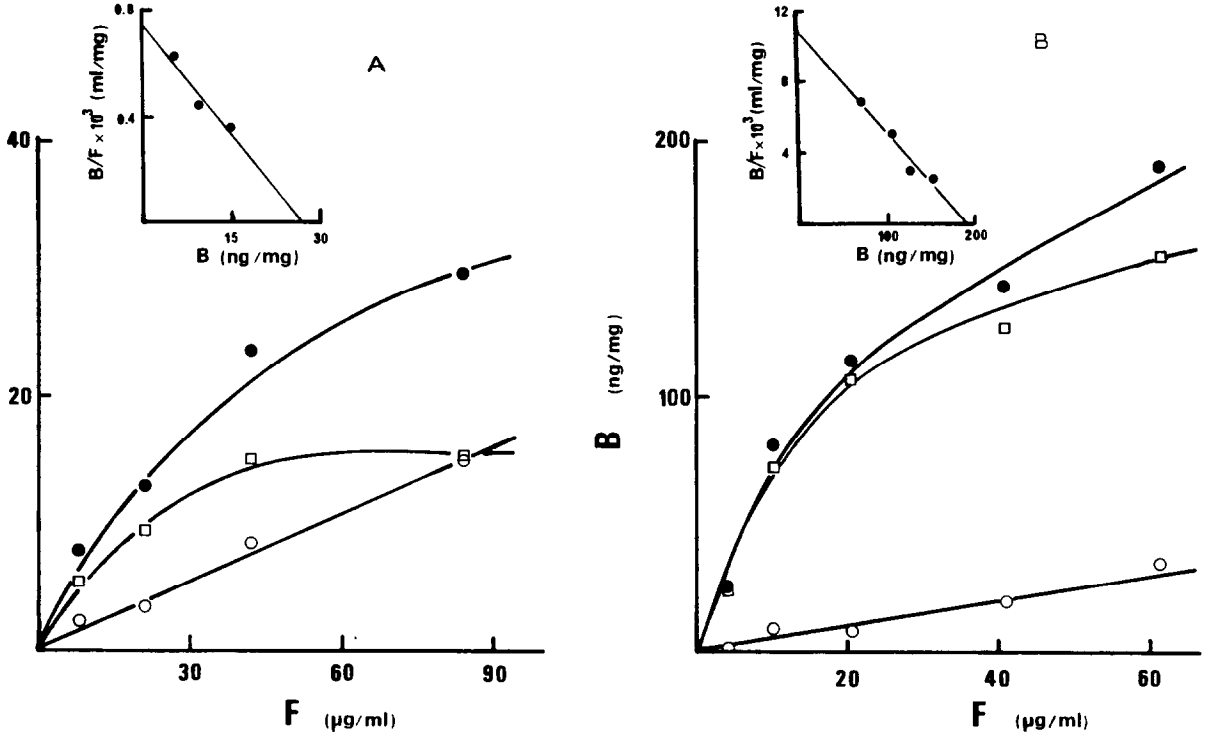

Fig.1. Binding of ^{125}I-Tf to normal (A) and L_2C (B) lymphocytes at 4°C. Binding was measured at the indicated concentrations, as described in section 2. (●) Total binding, (○) nonspecific binding, (□) specific binding.

case of normal lymphocytes. The nonspecific binding, obtained in presence of an excess of unlabeled Tf, was comparable in the two cell types.

The number of Tf receptors and their affinity for ^{125}I-Tf was determined for L_2C and normal cells by the method of Scatchard [14]. The apparent K_d calculated from the data in fig.1 was comparable for normal cells and L_2C lymphocytes (about 4×10^{-7} M), but the number of binding sites was greatly increased on the L_2C cell surface: about 8.5×10^4 receptors/cell (average of 3 experiments, ranging from 7.2 to 9.8×10^4) vs 8.2×10^3 receptors on normal cells.

Fig.2 shows the time course of ^{125}I-Tf binding to L_2C lymphocytes at 4 and 37°C. At both temperatures, the steady state was rapidly reached (about 10 min). As previously demonstrated, at 4°C, this corresponds to the Tf bound to the cell surface; at 37°C, the steady state reflects a balance between the amounts of ligand internalized and exocytosed [15]. The amount of Tf bound to the cell surface at 4°C amounted to about 40% of the ligand associated with cells at 37°C. When the uptake experiment was carried out in the presence of an excess of unlabeled Tf, the amount of ^{125}I-Tf associated with cells stayed constant with time, and decreased by more than 95%.

To discriminate between cell surface-bound and internalized Tf, we treated cells with an acid that releases surface-bound ligands. Fig.3 shows the total (uptake) and acid-resistant (internalized) amounts of ^{125}I-Tf associated with cells at 37°C, as a function of time. The difference between the two curves represents the fraction of the surface-bound ligand (about 40% of the total).

Fig.4 shows the results obtained in the pulse-chase experiment. The amount of surface-bound radioactivity diminished rapidly with incubation at 37°C, and during the first 5 min, about 50% of the surface-bound ^{125}I-Tf was internalized by cells. The amount of ^{125}I-Tf not associated with the cells increased with time, reflecting dissociation and exocytosis of the ^{125}I-Tf by cells.

Finally, we investigated the possibility that Tf and LDL receptors interact. For this purpose, we examined the binding and uptake of ^{125}I-Tf and

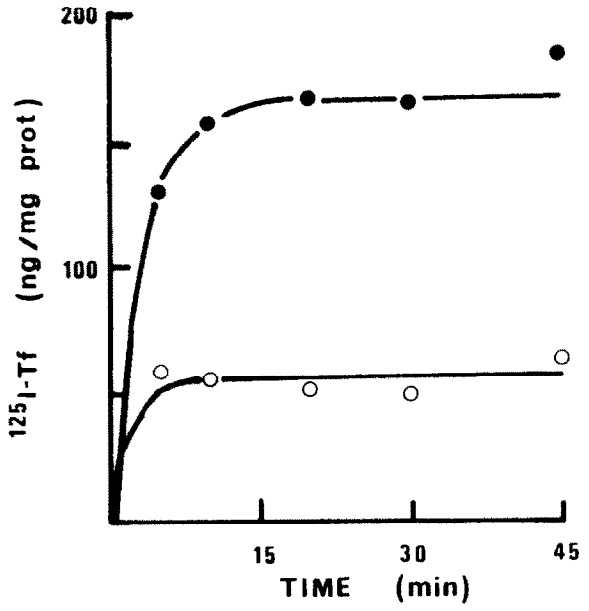

Fig.2. Time course of ^{125}I-Tf binding to L_2C lymphocytes. 5×10^6 cells were incubated with 40 μg/ml ^{125}I-Tf at either 4°C (○) or 37°C (●). All data points are averages of duplicate determinations and have been corrected for nonspecific binding (in the presence of 3 mg/ml unlabeled Tf).

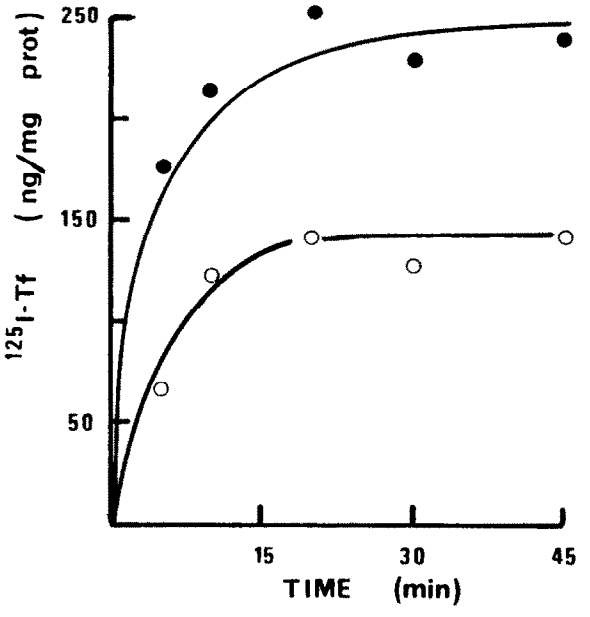

Fig.3. Time course of uptake (●) and internalization (○) of ^{125}I-Tf by L_2C lymphocytes at 37°C. 5×10^6 cells were incubated with 40 μg/ml ^{125}I-Tf at 37°C. At the indicated times, the binding buffer was removed and the cells were treated as described in section 2, except for internalization points (○), where the cells were pretreated with acetic acid.

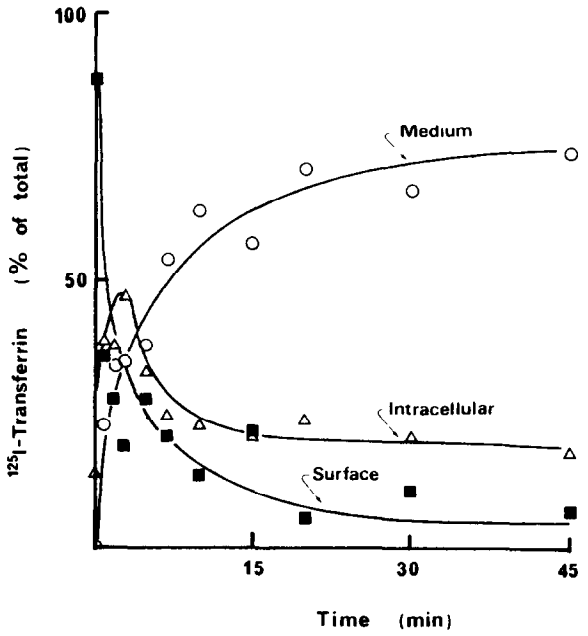

Fig.4. Diacytosis of ^{125}I-Tf in L_2C cells. ^{125}I-Tf was bound to cells for 2 h at 4°C, the excess of labeled ligand washed off, and the cells warmed to 37°C over different time periods, as described in section 2. Some tubes were treated with acid to determine intracellular (△) and surface (■) associated ligand. The amount of ligand not associated with the cells (○) was calculated from the quantity of ^{125}I-Tf present during the pulse-chase experiment minus the total uptake determined at each time.

^{125}I-LDL in the presence of an excess of LDL and Tf, respectively. Table 1 shows the results obtained in the uptake experiments. It can be seen that, in the case of both receptors, the amount of specific ligands taken up with time was not affected by an excess of another nonspecific ligand.

4. RESULTS

We have previously demonstrated that L_2C leukemia greatly increases the number of specific receptors for LDL on the lymphocyte surface [5]. Here, we show that the number of Tf receptors is also increased: 8200 sites/cell in normal lymphocytes vs 8.5×10^4 sites/cell in L_2C lymphocytes. This result confirms previous studies indicating the critical role of Tf and iron in the development of leukemia [16]. The dissociation constant is about the same for the two kinds of cells: $K_d = 4 \times 10^{-7}$ M. This value is higher than those generally observed in other cell types [7,12]. The discrepancy may be due to a lower affinity of human Tf for guinea pig lymphocytes than for homologous lymphocytes, as is the case for LDL [5].

Tf was rapidly internalized by L_2C cells at 37°C as shown in fig.3. The amount of Tf resistant to acid treatment increased with time and reached a steady state within 15 min. Thus, within 15 min, each internalized receptor was replaced on the cell surface by a recycled one. The levels of the steady states, with or without acid treatment (fig.3), can

Table 1

Uptake of ^{125}I-LDL and ^{125}I-Tf in the presence or absence of the nonspecific ligand (Tf and LDL, respectively), as a function of time

^{125}I-LDL uptake (ng/mg protein)			^{125}I-Tf uptake (ng/mg protein)		
Time (h)	Total	In the presence of Tf	Time (min)	Total	In the presence of LDL
1	149 ± 23	144 ± 55	5	264 ± 18	348 ± 15
2	455 ± 154	458 ± 51	10	376 ± 20	332 ± 54
3	426 ± 116	385 ± 115	15	330 ± 42	310 ± 47
4	413 ± 50	318 ± 27	30	332 ± 4	462 ± 104
6	425 ± 101	322 ± 68	45	288 ± 24	322 ± 32

Unlabeled ligands were added in the same excess (40-times the molar ratio). The data shown are means of 2 (^{125}I-Tf) or 3 (^{125}I-LDL) values of total uptake (i.e. specific + nonspecific uptake)

be used to calculate R_{in}/R_{out}, the ratio of the internal receptors to the surface receptors, which was equal to 1.5. 50% of the receptors entered the cells within 3–4 min (fig.4). This half-life ($T_{1/2}$), corresponds to a recycling time of about 10 min (estimated as described by Thilo [17] as: $(R_{in}/R_{out} + 1)T_{1/2}$), which is in good agreement with the literature [17].

These results demonstrate the efficiency of the Tf receptor on L_2C lymphocytes, in contrast with the weak internalization of LDL by these cells [5]. Thus, the difference between LDL and Tf internalization may be due to a specific impairment of the LDL receptor, rather than to a generalized membrane defect in L_2C cells. On the other hand, coated pits can be considered as segregation zones where specific receptors for different ligands are concentrated. If interactions between the different types of receptors exist at this level it could explain some dysfunctions, such as the weak internalization of LDL by L_2C lymphocytes. Although some receptors have been reported to be localized in the same coated pits in fibroblasts [18,19], it has been demonstrated that receptors for Tf, asialoglycoprotein and insulin are endocytosed independently in a hepatoma cell line [20]. We have verified that neither ligand, i.e. LDL or Tf, interfered with the binding of the other ligand to its specific receptor at 4°C (not shown). Moreover, experiments were carried out to investigate interactions in the internalization event (table 1). The results demonstrate that there is no interaction, either at the binding or internalization stage, between the receptors for LDL and Tf. Experiments investigating the impairment of the LDL receptor on L_2C cells have been carried out, and the results have been submitted for publication.

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REFERENCES

[1] Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1979) Nature 279, 679–685.
[2] Goldstein, J.L. and Brown, M.S. (1974) J. Biol. Chem. 254, 5133.5162.
[3] Ho, Y.K., Smith, R.G., Brown, M.S. and Goldstein, J.L. (1978) Blood 52, 1022–1114.
[4] Anderson, R.G.W., Brown, M.S. and Goldstein, J.L. (1981) J. Cell Biol. 88, 441–452.
[5] Sainte-Marie, J., Vidal, M., Philippot, J.R. and Bienvenue, A. (1985) J. Receptor Res. 5 (2&3), 171–192.
[6] Jandl, J.H., Inman, J.K., Simmons, R.L. and Allen, D.W. (1959) J. Clin. Invest. 38, 161–185.
[7] Karin, M. and Mintz, B. (1981) J. Biol. Chem. 256, 3245–3252.
[8] Newman, R., Schneider, C., Sutherland, R., Laida, V. and Greaves, M. (1982) Trends Biochem. Sci. 397–400.
[9] Congdon, C.D. and Lorenz, E. (1954) Am. J. Pathol. 30, 3245–3252.
[10] Chuillon Sainte-Marie, J., Authier, M.H., Cayzac, M. and Philippot, J.R. (1981) Eur. J. Biochem. 117, 219–224.
[11] Havel, R.J., Eder, H.A. and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345–1353.
[12] Klausner, R.D., Van Renswoude, J., Ashwell, G., Kempf, C., Schechter, A.N., Dean, A. and Bridges, K.R. (1983) J. Biol. Chem. 258, 4715–4724.
[13] Lowry, O.H., Rosebrough, M.J., Farr, A.L. and Randall, R.J. (1951) Anal. Biochem. 110, 165–170.
[14] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672.
[15] Bretscher, M.S. (1982) in: Membrane Recycling (Ciba Foundation Symposium no. 92) pp. 115–119, Pitman, London.
[16] Taetle, R., Rhyner, K., Castagnola, J., To Dong and Mendelsohn, J. (1985) J. Clin. Invest. 75, 1061–1067.
[17] Thilo, L. (1985) Biochim. Biophys. Acta 822, 243–266.
[18] Via, D.P., Willingham, M.C., Pastan, I., Gotto, A.M. and Smith, L.C. (1982) Exp. Cell Res. 141, 15–22.
[19] Carpentier, J.-L., Gorden, P., Anderson, R.G.W., Goldstein, J.L., Brown, M.S., Cohen, S. and Orci, L. (1982) J. Cell Biol. 95, 73–77.
[20] Ciechanover, A., Schwartz, A.L. and Lodish, H.F. (1983) J. Cell Biochem. 23, 107–130.