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# Emergence of Insulin Receptors on Human Lymphocytes During *In Vitro* Transformation

**(concanavalin A/cell growth and division/insulin receptors)**

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**ABSTRACT** **Essentially no specific binding sites for insulin are detected in small lymphocytes freshly isolated from human blood. Insulin-binding sites appear on the lymphocyte surface during transformation *in vitro* with concanavalin A, and the number of these receptors increases sharply to reach a maximum between 24 and 46 hr after exposure to the mitogen. The appearance of *de novo* binding sites for insulin coincides with the increase in [$^3$H]thymidine uptake into nuclear DNA and clearly precedes the appearance of enlarged, morphologically transformed cells. No changes in insulin-binding are detected in unstimulated control cultures. A maximum of about 350 molecules of insulin can bind per transformed lymphocyte, while less than six insulin molecules bind to an untransformed cell. Circulating human leukemic lymphoblasts bind about as much insulin as the lymphocytes transformed *in vitro*. Giant, polynucleated, transformed lymphocytes cultured in the presence of cytochalasin B bind about 10 times more insulin than transformed lymphocytes, which is in harmony with a 10-fold increase in cell-surface area in these cells. Specific binding of insulin is a saturable process in transformed lymphocytes but not in the untransformed cells. In transformed cells, [$^{125}$I]-insulin is displaced by as little as 2 ng/ml of native insulin, while in untransformed cells no significant displacement is observed with native insulin. Digestion of transformed cells with phospholipase C (EC 3.1.4.3.) enhances the specific binding of [$^{125}$I]insulin 3-fold, but no effect occurs with untransformed cells. These observations indicate a possible functional role of insulin and of adenylate cyclase in cell growth and division.**

The binding of insulin to its receptor sites on fat cells and on liver and fat-cell membranes has been studied extensively (1–8). Insulin-receptor sites on cells other than the typical target cells for insulin, namely human circulating cells and cultured fibroblasts, have recently been described (9). The present studies demonstrate that while no significant specific binding of insulin is detectable on normal peripheral blood lymphocytes of humans, a dramatic appearance of insulin receptors occurs during lymphocyte transformation induced by the plant mitogen concanavalin A. The short-term lymphocyte culture provides a useful model for investigation of changes in the number and properties of surface receptor sites of cells as they undergo functional changes such as metabolic activation, differentiation, and cell division. The present studies are also of interest in view of the profound insulin-like biological properties of concanavalin A (10), the inhibition by insulin (11, *) and by concanavalin A (10, *) of adenylate cyclase activity in isolated membrane preparations, the growth-promoting properties of insulin in various mammalian cells in tissue culture (12, 15), and the important relationships that apparently exist between cyclic AMP and cell growth (15–18).

* Tell, G. & Cuatrecases, P., submitted to *J. Biol. Chem.*

## METHODS

Lymphocytes were separated from other leukocytes by passage of freshly drawn, heparinized blood (200–500 ml) from a healthy donor through a sterile, disposable nylon-fiber column (Fenwal Laboratories) that was immediately prewashed (at 37°) with 250 ml of sterile normal saline. The erythrocytes (in 10-ml aliquots) were allowed to settle by gravity at 37° and the lymphocyte-rich plasma was collected before the appearance of a visible buffy coat. For removal of excess platelets, the plasma was centrifuged at 150 × *g* for 15 min, and the pellet was washed 2–3 times with Medium 199 containing 5% fetal-bovine serum. The lymphocytes, recovered in 60% yield, constituted 95–98% of the cells; 1–5% were granulocytes and less than 1% were monocytes. Platelets did not exceed 50, and erythrocytes did not exceed 200 per 100 leukocytes. Leukocyte counts (modified Neubauer hemocytometer) were based on a count of at least 700 cells. For differential counts (500 cells, three slides), samples on pulled coverslips were fixed in 99% methanol and stained with Giemsa stain. Increased cell size, characteristic nuclear staining, multiple nucleoli, and cytoplasmic basophilia were used to judge blastic transformation.

For culture the cells were suspended in Medium 199 supplemented with 8% fresh homologous serum containing 100 units of penicillin and 50 μg of streptomycin per ml. The initial cell density was 5 to 7 × $10^5$ per ml for purified lymphocytes and 1.2 to 1.6 × $10^6$ per ml for unfractionated leukocytes. After addition of concanvalin A (40 μg/ml), the cultures were incubated at 37° in a controlled atmosphere at 5% $CO_2$ in humidified air.

Enlarged polynucleated lymphocytes were obtained by culturing lymphocytes with 6 μg/ml of cytochalasin B for 6 days; about 60% of the cells had two or more nuclei. The cells were washed 3 times with Hanks' balanced salt solution–0.1% albumin and centrifuged at 30 to 50 × *g* for removal of debris and smaller cells. About 85% of the cells were enlarged and nearly all had more than 2 nuclei.

The specific binding of [$^{125}$I]insulin (0.9 Ci/μmol) to lymphocytes was determined by procedures described (1, 3). After

washing twice at 24° with Hanks' balanced salt solution–0.1% albumin, the lymphocytes (3 to 8 × $10^6$ cells) were incubated with vigorous shaking in polystyrene tubes for 40 min at 24° in 0.2 ml of the same buffer containing 0.35–0.7 nM [$^{125}$I]insulin; native insulin (5 μg/ml) was added to control samples to correct for nonspecific binding (1, 3).

## RESULTS

### Changes in the binding of insulin to lymphocytes during transformation

Transformed lymphocytes begin to appear between 24 and 30 hr after addition of concanavalin A (Fig. 1, *upper*). They increase in number rapidly after about 48 hr, and a plateau is reached at a critical cell density after about 80 hr. At this time about 65–90% of all lymphocytes are transformed cells, depending on the blood donor. If concanavalin A is omitted from the culture medium (Fig. 1, *lower*), less than 3% of the lymphocytes undergo spontaneous transformation, and the cell density does not increase.

In parallel with these changes, few if any receptor sites for insulin are found in cells harvested from stimulated cultures during the first 1.5 days. On the second and third days in culture insulin-binding increases sharply, reaching a maximum at the time of maximal lymphocyte transformation. No changes occur in the binding of insulin to unstimulated control cells in culture.

When 95–98% pure lymphocyte preparations are used for culture, and when insulin-binding is corrected for changes in cell density, virtually no receptor sites for insulin are detected within 24 hr after addition of concanavalin A (Fig. 2). The

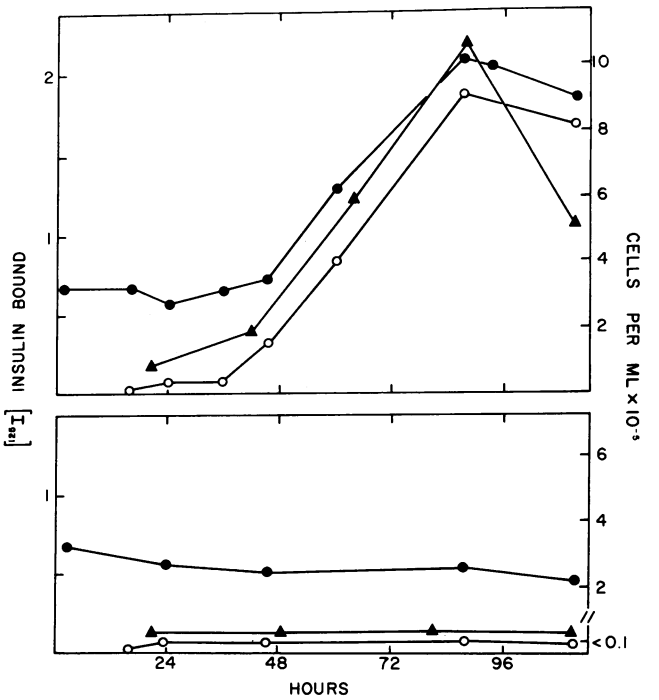


FIG. 1. Growth pattern of human lymphocytes in culture correlated with their capacity to specifically bind insulin. Unfractionated leukocytes (1.6 × $10^6$ cells/ml) were cultured with (*upper*) or without (*lower*) concanavalin A (40 μg/ml). The total number, per ml, of lymphocytes (●) and of morphologically transformed cells (○) is described. The specific binding of [$^{125}$I]insulin is given as $10^{16}$ × mol/ml of culture (▲).

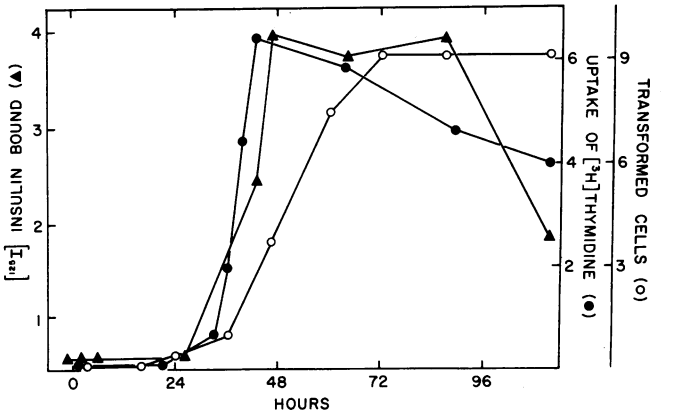


FIG. 2. Binding of insulin to lymphocytes during transformation. Column-purified human peripheral-blood lymphocytes (5 × $10^5$ cells/ml) were cultured with concanavalin A (40 μg/ml). Specific binding of [$^{125}$I]insulin ($10^{16}$ × mol), incorporation of [$^3$H]thymidine into DNA ($10^4$ × cpm), and the number of transformed cells (×$10^5$) are expressed per $10^6$ cells. For thymidine incorporation into nuclear DNA, samples were incubated with 1 μCi/ml [$^3$H]thymidine (6.7 mCi/μmol) for 3 hr at 37° in 5% $CO_2$ in air. The cells, chilled and washed with cold saline, were precipitated with 5% $Cl_3CCOOH$. The precipitate, washed with cold 5% $Cl_3CCOOH$ and methanol, was dissolved (15 min at 70°) in hydroxide of hyamine before counting. [$^3$H]Thymidine uptake by unstimulated control culture cells was always less than 500 cpm per $10^6$ cells and was therefore neglected. All data represent mean values of three determinations.

number of receptors increases sharply after 24 hr, and a plateau is reached by 46 hr. These changes in the insulin receptor coincide closely with the rapid increase in the incorporation of [$^3$H]thymidine into nuclear DNA, and they clearly precede the appearance of blast-like cells or the increase in the number of cells. After 4–5 days insulin-binding decreases in parallel with a decrease in DNA synthesis and in cell density, which indicates defective proliferative capacity of the cells and the beginning of cell death.

### Binding of insulin to contaminating cells other than lymphocytes

It is unlikely that insulin-binding to granulocytes is of significance in the present studies. These cells decay rapidly *in vitro* under the conditions used to culture lymphocytes. Even with unfractionated leukocytes the number of granulocytes decreases to less than 5 in 100 leukocytes within the first 48 hr of culture. Furthermore, column-purified leukocytes contain fewer than 5 granulocytes per 100 leukocytes.

Rat peritoneal macrophages bind approximately as much insulin as transformed lymphocytes. Most blood macrophages are eliminated by passage through nylon-fiber columns, but the few that remain can undergo cell division in the lymphocyte culture. The number of these cells, however, never exceeded 8 per 100 leukocytes by the third or fourth day of culture.

No significant specific binding of insulin to platelets and erythrocytes is detected when they are tested in concentrations 10 and 20 times greater than those that maximally contaminate the lymphocytes used in these experiments.

Granulocytes, blood macrophages, erythrocytes, and blood platelets, thus, do not contribute significantly to the specific

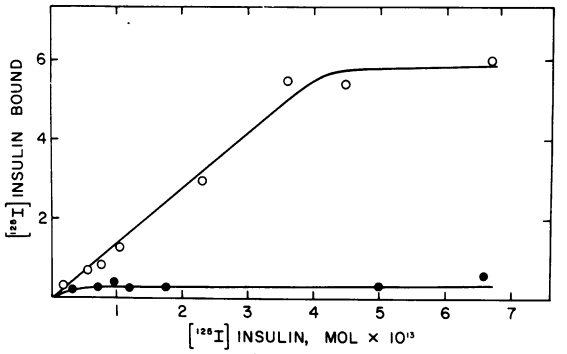


FIG. 3. Specific binding of insulin to transformed (○) and untransformed (●) lymphocytes as a function of [$^{125}$I]insulin concentration. Cells (3 to 4 × $10^6$) were incubated (40 min, 24°) in 0.2 ml of Hanks' balanced salt solution–0.1% albumin with [$^{125}$I]insulin in the presence and absence of native insulin (5 μg/ml). Insulin bound is in $10^{16}$ × mol per $10^6$ cells.

binding of [$^{125}$I]insulin measured in the present experiments. This is substantiated by the absence of changes in insulin-binding in unstimulated control cultures (Fig. 1, *lower*), which differ from the stimulated cultures only by the absence of lymphocyte transformation.

**Specific binding of insulin exists in transformed but not in untransformed lymphocytes**

The specific binding of insulin to transformed lymphocytes is saturable with respect to the concentration of [$^{125}$I]insulin (Fig. 3). A maximum of about 5 × $10^{-16}$ mol of insulin are bound to $10^6$ cells (about 350 molecules of insulin per cell). Half-maximal saturation occurs with about 1 nM insulin.

In contrast, untransformed lymphocytes bind a maximum of 3 × $10^{-17}$ mol of insulin per $10^6$ cells (Fig. 3), or about 20 molecules of insulin per cell. This low amount of "specific" binding does not vary with the concentration of [$^{125}$I]insulin used. This indicates that if a true receptor saturation curve does exist in these cells, the binding must be of extraordinarily high affinity. Another notable observation is that increasing the concentration of untransformed cells by 3-fold does not

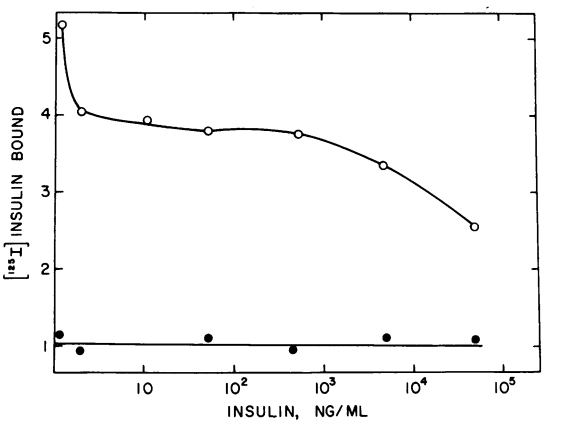


FIG. 4. Effect of native insulin on the binding of [$^{125}$I]insulin to transformed (○; 5 × $10^6$ cells) and untransformed (●; 8 × $10^6$ cells) lymphocytes incubated in 0.2 ml of buffer containing 2.5 nM [$^{125}$I]insulin as described in Fig. 3. Insulin bound is in $10^{16}$ × mol per $10^6$ cells.

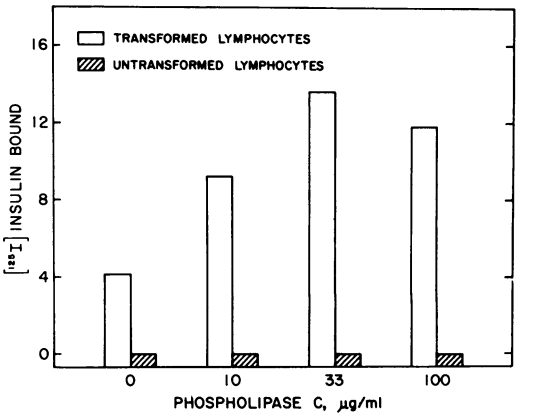


FIG. 5. Effect of digestion of lymphocytes with phospholipase C from *Clostridium perfringens* on the specific binding of [$^{125}$I]-insulin. Cells (5 × $10^6$) were incubated for 30 min at 37° in 0.2 ml of Hanks' balanced salt solution–0.1% albumin containing increasing amounts of enzyme before testing for specific binding of [$^{125}$I]insulin ($10^{16}$ × mol per 5 × $10^6$ cells) as described in Figs. 3 and 4.

increase the amount of "specific" binding of insulin to these cells (Fig. 4). Under these conditions untransformed cells must bind less than six molecules of insulin per cell.

If the small amount of binding of insulin to untransformed cells represents binding to receptors it must reflect an interaction of unusually high affinity (Fig. 3). This is not the case, since native insulin in concentrations (2 ng to 50 μg/ml) that effectively displace the binding of [$^{125}$I]insulin to transformed cells is without effect on untransformed cells (Fig. 4). The small amount of binding of insulin to untransformed cells almost certainly does not represent binding to true receptors and must therefore be considered as "nonspecific."

**Unmasking of insulin-binding sites by phospholipase C (EC 3.1.4.3.) digestion**

Perturbation of phospholipids of liver (2, 4) and fat-cell (4) membranes by procedures such as digestion with phospholipase C or A results in the appearance of new binding sites for insulin. To determine if the apparent lack of insulin receptors in untransformed cells, and if the gradual appearance of these receptors during the process of transformation, represent only differences in the exposure or accessibility of binding structures already present in the membrane, insulin-binding was examined after digestion of lymphocytes with phospholipase C (Fig. 5). As in other tissues (2, 4), digestion with this enzyme increases the insulin-binding capacity of lymphocytes, but this occurs only with transformed lymphocytes. This unmasking effect is increasingly marked during the progress of transformation and parallels closely the emergence of insulin-binding described in Figs. 1 and 2. Thus, the change in insulin-binding probably reflects *de novo* synthesis or assembly of receptor structures rather than a simple relocation of already present but masked receptors.

**Binding of insulin to lymphocytes cultured with cytochalasin B**

Cytochalasin B, a fungal metabolite, induces the formation of multinucleated fibroblasts (19, 20) and lymphocytes (21, 22) by blocking cytoplasmic cleavage after nuclear division.

The large, multinucleated lymphocytes cultured (6 days) in the presence of cytochalasin B (see *Methods*) can specifically bind about $2.4 \times 10^{-15}$ mol of insulin per cell, or about 10 times more than the control, transformed lymphocytes. This difference does not represent a change in the density of the cell surface receptors for insulin, since the mean diameter of the multinucleate cells is 3 times greater than that of the control cells, which corresponds to a 9-fold increase in surface area. It is of interest that at least one expression of transformation (the emergence of insulin receptors) does not depend on the separation of daughter cells after cell division. Furthermore, these studies suggest that important functional and topographic features of the surfaces of such cells may be relatively intact.

**Specific binding of insulin to leukemic lymphoblasts**

Leukemic lymphoblasts (99.5% pure), prepared from the peripheral blood of a patient with acute lymphocytic leukemia who had not received chemotherapy for the preceding month, bind approximately the same amount of insulin as do lymphocytes transformed *in vitro* with concanavalin A. Similarly, human lymphocytes (RPMI 6237, Associated Biomedic Systems) derived from normal peripheral blood and maintained in long-term culture over many generations possess comparable numbers of insulin receptors.

## DISCUSSION

The *de novo* appearance of insulin receptors is not an early event in the process of lymphocyte transformation. It is not associated with the initial activation of cellular RNA and protein synthesis since these are known to occur within the first 24 hr of lymphocyte transformation. The appearance of insulin receptors coincides closely with changes that occur in the uptake of [$^3$H]thymidine and with the morphological changes characteristic of transformation. Cell enlargement is clearly delayed by 6–12 hr compared to the increase in insulin-binding and [$^3$H]thymidine incorporation. It is thus quite possible that the emergent insulin receptors are a prerequisite for, rather than a consequence of, cell enlargement and subsequent cell division.

The increasing numbers of blast-like cells during culture is the result of transformation of small lymphocytes as well as of division of already transformed cells. Between 24 and 48 hr after exposure to concanavalin A, the predominant change appears to be blastic transformation rather than cell division. After about 48 hr, however, the rapid rise in the number of cells indicates that now a substantial proportion of the cells are undergoing cell division. Since by this time the changes occurring in insulin-binding are nearly complete, it is very likely that the increase of insulin receptors occurs during the process of transformation itself and is not dependent on cell division.

Scott and Marchesi (23) have described a rapid increase in the density of intramembranous particles (seen by freeze-etching) of lymphocytes that begins about 24 hr after exposure to phytohemagglutinin. They suggested that these changes may represent an increase in the synthesis and insertion of membrane-glycoprotein components, such as receptors and membrane antigens, occurring during transformation. It will be interesting to determine if the insulin receptors are localized with these membranous particles, and to investigate possible changes in other membrane-localized antigens and receptors during transformation. Attempts to demonstrate significant specific binding of glucagon to transformed or untransformed cells by the procedures used for insulin have not been successful.

TABLE 1. *Density of insulin-binding sites on the cell surface*

| Cell type | Mean cell-surface area ($\mu m^2$) | Insulin-binding sites per cell | Insulin-binding sites per $\mu m^2$ |
|---|---|---|---|
| Untransformed lymphocytes | 130 | <6 | <0.05 |
| Transformed lymphocytes (and leukemic lymphoblasts) | 1,400 | 350 | 0.3 |
| Polynucleated, transformed lymphocytes (cytochalasin B) | 12,700 | 3,500 | 0.3 |
| Isolated fat cells, rat (ref. 1) | 5,000 | 11,000 | 2.2 |

The number and density of insulin-binding sites on the surface of various cells is summarized in Table 1. The present studies indicate quite strongly that untransformed, normal human lymphocytes possess virtually no specific receptors for insulin. Therefore, contrary to an earlier suggestion (9), these cells are inadequate for examination of the properties of the human insulin receptor in endocrine disorders. The binding of insulin to human lymphocytes, may, however, prove useful in the study of disorders characterized by abnormal growth patterns, as occurs in neoplasia.

It has not been possible in this laboratory to detect unequivocal biological effects (glucose oxidation or transport, glycogen synthesis) of insulin on untransformed lymphocytes. It can be readily demonstrated, however, that physiological concentrations (10–100 pM) of insulin inhibit adenylate cyclase activity that is stimulated by epinephrine in isolated membranes of cultured human lymphocytes (G. Tell and P. Cuatrecasas, unpublished), consistent with the presence of true receptors in these cells.

It is interesting to consider the possible role of insulin in processes of cell growth and division. It has been demonstrated that insulin has major growth-promoting properties in various cells in culture (12–15). It is also well established that serum contains essential growth-promoting properties that can be replaced by insulin under some circumstances. The present studies on lymphocyte transformation indicate that insulin, at physiological concentrations, cannot be required initially since the receptors for this hormone are absent. The initial requirement for serum therefore reflects a requirement for a different factor. A possible requirement for insulin at later stages, perhaps preceding cell growth and division, cannot, however, be easily dismissed.

It is pertinent that concanavalin A has very potent insulin-like properties in various physiological processes (10). Concanavalin A, like insulin, also inhibits adenylate cyclase activity of isolated membrane preparations at concentrations that cause lymphocyte transformation (10, *). Furthermore, concentrations of the lectin that are toxic to lymphocytes actually stimulate the activity of adenylate cyclase (*). It is

possible that the "insulin-like" property (adenylate cyclase inhibition) of the plant lectin is a fundamental biochemical event in transformation. The mitogen could initiate "insulin-like" effects in cells lacking receptors for the hormone. It is known that the presence of concanavalin A is required only during the first 24 hr of lymphocyte transformation (24), which corresponds with the time required for the emergence of insulin receptors. It will be interesting to determine if other mitogenic substances can also mimic the biological effects of insulin, and in particular, whether such stimuli can also depress the activity of adenylate cyclase. It is notable that Smith *et al.* (25) have reported that compounds that enhance the concentration of lymphocyte cyclic AMP (aminophylline, isoproterenol, prostaglandins), as well as dibutyryl cyclic AMP, markedly inhibit human lymphocyte transformation by phytohemagglutinin. Furthermore, the concentration of cyclic AMP is decreased in lymphocytes exposed to this mitogen for 24 hr (26). These considerations are especially important in view of the recent recognition that significant relationships exist between cyclic AMP and cell growth (15–18).

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