port of metals. Because of this complexity, it is not surprising that earlier experiments with lead and milk should have yielded conflicting results (*6*).

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Binding and Mobility of the Cell Surface Receptors for 3,3′,5-Triiodo-L-Thyronine

Abstract. *A fluorescent derivative of the thyroid hormone 3,3′,5-triiodo-L-thyronine binds to cultured mouse fibroblasts; such binding is saturable. Video intensification fluorescence microscopy indicates that binding occurs at the plasma membrane. Diffusion coefficients, obtained by fluorescence photobleaching recovery, are consistent with binding to a protein receptor on the cell surface.*

Although much evidence suggests that thyroid hormone action is initiated by binding of 3,3′,5-triiodo-L-thyronine (T_3) to nuclear receptors (*1*), the mechanism for delivery of T_3 to the nucleus is not fully understood. It was long thought that T_3 enters cells by passive diffusion through the plasma membrane (*2*), but recent studies with [^{125}I]T_3 suggest that there are cell-surface receptors for T_3 and that entry of T_3 is, at least in part, energy-dependent (*3*). We have synthesized a rhodamine derivative of T_3 (Rho-T_3) that binds specifically to the nuclear receptor for T_3 with a dissociation constant (K_d) of 20 n*M* (*4*), and in this report we show that Rho-T_3 also binds to a membrane component that has a mobility in the plasma membrane similar to the mobility of polypeptide hormone receptors.

When 3T3 fibroblasts from Swiss albino mice are incubated with Rho-T_3 for 20 minutes at 23° or 37°C, fluorescence is rapidly localized in endocytic vesicles that appear as bright points of light (Fig. 1E) (*5*). Only background fluorescence is observed in cells incubated with rhodamine-thyronine (Rho-T_0). In double-labeling experiments with fluorescein-labeled α_2-macroglobulin and Rho-T_3, we found that these endocytic vesicles are the same as those which take up insulin, epidermal growth factor, and the serum protein α_2-macroglobulin (*5*). We demonstrated previously (*6*) that α_2-macroglobulin-occupied receptors cluster over coated pits before they are internalized and that the clustering can be inhibited by primary alkylamines. Figure 1 shows that methylamine also affects the uptake of Rho-T_3. On a flat cell (Fig. 1A) the fluorescence appears diffusely distributed after 20 minutes of incubation with Rho-T_3 at 23°C in the presence of 20 m*M* methylamine. By focusing up and down through the cell we found that the fluorescence was located at or near the upper and lower surfaces of the cell, as would be expected for binding to the plasma membrane. When grown at high density the cells become somewhat rounded, and this allows one to see clear evidence for membrane fluorescence because the optical path at the boundary between cells contains more of the plasma membrane than the optical path away from the boundary. Whereas bright fluorescence occurs at the boundary between two cells after incubation with Rho-T_3 in the presence of 20 m*M* methylamine, such incubation in the absence of methylamine leads to fluorescence appearing in endocytic vesicles but not, at least in quantity, at the plasma membrane (Fig. 1, C and E).

To show that binding was saturable, we used a microscope fluorescence spectrophotometer to quantify the fluorescence intensity observed after incubating the cells with Rho-T_3 and methylamine in the presence or absence of unlabeled T_3 (20 μ*M*) (see legend to Fig. 1). Fluorescence intensities were measured on 20 randomly selected cells in each dish (*7*). Untreated cells gave an average fluorescence intensity of 33 arbitrary units ± 7 (standard deviation); this intensity was due to cellular autofluorescence. Cells treated with Rho-T_3 alone had an intensity of 103 ± 42 units and cells incubated with Rho-T_3 and excess unlabeled T_3 had an intensity of 54 ± 13 units. Thus, unlabeled T_3 competed for 70 percent of the Rho-T_3 binding. The standard deviations in these measurements are an indication of cellular heterogeneity. Duplicate dishes gave the same average values within ± 10 percent.

Since Rho-T_3 remains associated with the plasma membrane in the presence of methylamine, we were able to measure the diffusion coefficient, *D*, in the plane of the plasma membrane using fluorescence photobleaching recovery (FPR). In this method (*8*), fluorescence from a small region (approximately 1 μm in diameter) on the plasma membrane is partially bleached by a brief exposure to laser light focused on the membrane. The diffusion coefficient is determined from the rate at which fluorescent molecules diffuse into the bleached area. In general, the diffusion coefficients of membrane proteins are 5×10^{-10} cm^2/sec or less, whereas the diffusion coefficients of lipids and lipid probes are about 10^{-8} cm^2/sec (*9*). By using FPR we could determine whether Rho-T_3 behaves as if it is simply dissolved in the membrane lipid phase and diffuses like a lipid probe, or whether its diffusion is more characteristic of a membrane protein. As shown in

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Table 1. Lateral diffusion coefficients determined for Rho-T_3 and other membrane-associated molecules on 3T3 cells by fluorescence photobleaching recovery (*8*).

Ligand	D (cm^2/sec) $\times 10^{10}$	Fractional recovery (%)	Reference
Rho-T_3	2.8 ± 1.5	69 ± 29	This report
Rho-insulin	4.8 ± 1.6	50 to 80	(*11*)
Rho-epidermal growth factor	3.4 ± 0.5	50 to 85	(*11*)
Rho-α_2-macroglobulin	7.8 ± 3.5	53 ± 7	(*12*)
DiI*	100	~100	(*11*)

*3,3'-Dioctadecylindocarbocyanine iodide, a fluorescent lipid probe.

Table 1, the diffusion coefficient of $(2.8 \pm 1.5) \times 10^{-10}$ cm^2/sec for Rho-T_3 was indistinguishable from the diffusion coefficients previously measured for the rhodamine derivatives of insulin, epidermal growth factor, and α_2-macroglobulin on these cells. This, together with the saturability and specificity of the binding, suggests that Rho-T_3 binds to a cell-surface protein receptor on 3T3 cells. The similarities observed in the uptake of Rho-T_3, Rho-α_2-macroglobulin, Rho-insulin, and Rho-epidermal growth factor also suggest that a cell-surface protein receptor is involved in the uptake of Rho-T_3.

We have not observed Rho-T_3 fluorescence in nuclei, perhaps because of a change in the fluorescent properties of Rho-T_3 after it binds to the nuclear receptor. Alternatively, the T_3 receptors may be diffusely distributed in the nucleus, making them difficult to observe against the cytoplasmic fluorescence.

The physiological significance of binding to a cell surface receptor and receptor-mediated uptake of T_3 is not clear. Although 70 percent of the Rho-T_3 binding could be blocked by unlabeled T_3, only 20 percent of [^{125}I]T_3 uptake could be blocked by Rho-T_3 (*5*). This raises the possibilities that Rho-T_3 has a decreased affinity for the receptor and thus is unable to compete effectively with T_3, or that there is more than one mechanism of uptake of T_3 into the cells and Rho-T_3 can use only one of these mechanisms (that is, receptor-mediated uptake). The consequences of T_3 accumulating in endocytic vesicles are also unclear. We have observed (data not shown) that vesicles containing Rho-T_3 or α_2-macroglobulin (*10*) undergo rapid saltatory motion inside the cell. These vesicles might be responsible for delivering T_3 rapidly to other locations in the cell.

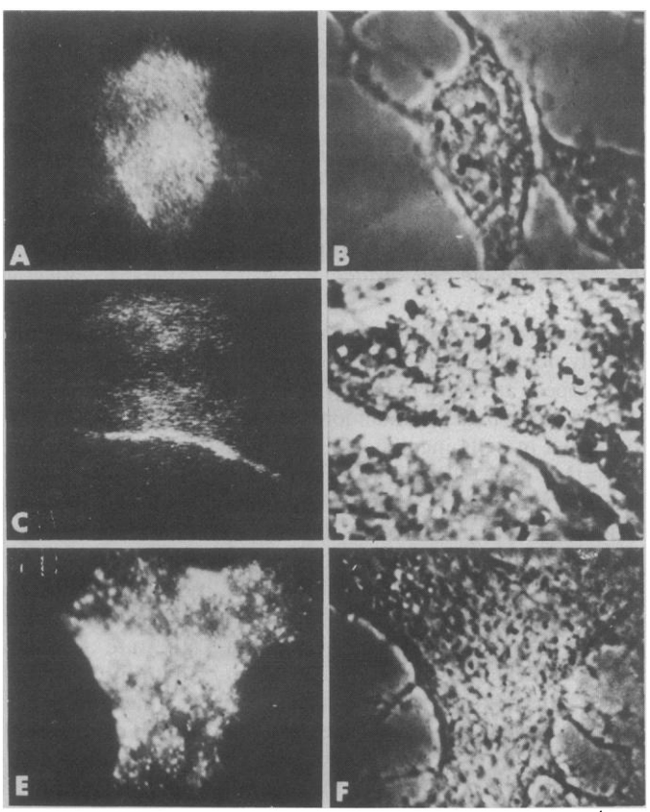

Fig. 1. Binding of Rho-T_3 to 3T3 cells. The cells were rinsed five times with Dulbecco-Vogt's modified Eagle's medium and then incubated with $10^{-7}M$ Rho-T_3 for 20 minutes at 23°C. When present, methylamine (20 m*M*) was added 30 minutes before the addition of Rho-T_3. After incubation, the cells were rinsed five times with serum-free medium, three times with serum-free medium containing bovine serum albumin (1 mg/ml), and five times with phosphate-buffered saline (each 1 ml). The cells were then observed with a silicon intensifier target television camera mounted on a Zeiss standard epifluorescence microscope with a × 63 (N.A. 1.4) objective as described (*10*). (A, C, and E) Fluorescence micrographs; (B, D, and F) the same fields under phase-contrast illumination. (A and B) A flat cell incubated with Rho-T_3 in the presence of methylamine shows a diffuse pattern of fluorescence. (C and D) Cells incubated with Rho-T_3 in the presence of methylamine show a heavy concentration of fluorescence at the boundary between cells. (E and F) Cells incubated with Rho-T_3 without methylamine show the concentration of Rho-T_3 in endocytic vesicles (magnification: × 2500 in A, B, E, and F; × 800 in C and D).

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Differentiation of Murine Bone Marrow Stem Cells in vitro: Long-Term Growth Promoted by a Lymphocyte-Derived Mediator

Abstract. *In attempts to induce differentiation of lymphoid cells from hematopoietic stem cells in vitro, the effects of allogeneic effect factor on the growth of murine bone marrow cultures were studied. Allogeneic effect factor is a soluble mediator derived from mixed secondary murine leukocyte cultures. For several weeks it supported the growth of bone marrow cultures, as indicated by the maintenance of stem cell activity, cellular proliferation, and heterogeneity. Another lymphokine, T cell growth factor, did not. Pre-T lymphocytes could be detected in these cultures for several weeks.*

Bone marrow is a source of pluripotent hematopoietic stem cells that can give rise to all the cellular elements of the blood, including myeloid cells, erythrocytes, and lymphocytes (*1*). The study of the pathways and regulatory mechanisms involved in hematopoiesis has been greatly aided by the development of a tissue culture system that supports the growth and differentiation of the pluripotent stem cells of mice (*2*) and tree shrews (*3*) for many weeks. This system has made it possible to induce myeloid cells, megakaryocytes, and erythrocytes to differentiate from their precursors in vitro (*4*). However, differentiation of lymphoid cells was not observed in these cultures until very recently, although cells derived from long-term cultures of murine bone marrow were found to be capable of reconstituting the lymphoid tissues of irradiated recipients and differentiating into immunocompetent lymphocytes in vivo (*5*). While it has not been possible to maintain stem cell activity in long-term cultures of human bone marrow (*3*), sustained growth of thymus-derived (T) lymphocytes from human bone marrow was accomplished by using conditioned medium of mitogen-stimulated T lymphocyte cultures containing the lymphokine T cell growth factor (TCGF) (*6*). The development of techniques for inducing immunocompetent lymphoid cells to differentiate from pluripotent stem cells in vitro would greatly facilitate studies of the generation of the immune system and its diversity during ontogenesis.

It was recently reported that Thy-1-bearing T lymphocytes can be generated in murine bone marrow cultures with or without a stimulus by TCGF (*7*). Such cells were generated in conventional Dexter-type cultures, which require that an adherent layer of bone marrow cells be established first, followed 2 to 3 weeks later by a second inoculum of fresh bone marrow cells, in order to obtain long-term maintenance of stem cells (*2*).

For several years our laboratory has studied an immunoregulatory soluble mediator termed allogeneic effect factor (AEF), which is a product of a secondary mixed lymphocyte culture of T cells previously activated to alloantigens in vivo (*8*). Allogeneic effect factor is a glycoprotein composed of two subunits (40,000 and 12,000 daltons) and possesses Ia antigen, which is determined by the I region of the murine *H-2* major histocompatibility complex, and β_2-microglobulin determinants (*9*). Biologically, AEF induces differentiation of bone marrow-derived (B) lymphocytes to mature antibody-secreting cells (*7, 8*) and also exerts proliferative and differentiating influences on T lymphocytes. It stimulates normal T cells to develop, in the absence of exogenous antigens, into cytotoxic T lymphocytes that preferentially lyse *H-2*-identical target cells and into effector cells capable of responding to irradiated syngeneic target cells in secondary mixed lymphocyte reactions (*10*). This is in contrast to TCGF, which acts on antigen- or mitogen-activated T cells but not on normal unstimulated T cells, and thus acts only as a second signal for such cells (*11*).

These unique effects of AEF on T lymphocytes (*10*) suggest that AEF-containing supernatants may affect the differentiation of murine hematopoietic stem cells in vitro, particularly along the lymphoid line. This report describes initial investigations of this possibility. (Hereafter, the term AEF refers to AEF-containing culture supernatants that also contain a heterogeneous collection of other molecules.)

Bone marrow cultures were established in Eagle's minimum essential medium (Dulbecco's modification) supplemented with 20 percent horse serum. To test the effects of AEF on the growth of bone marrow cells, we deliberately selected conditions that resulted in the rapid decline and loss of cell function in control cultures not supplemented with AEF. These suboptimal conditions in-

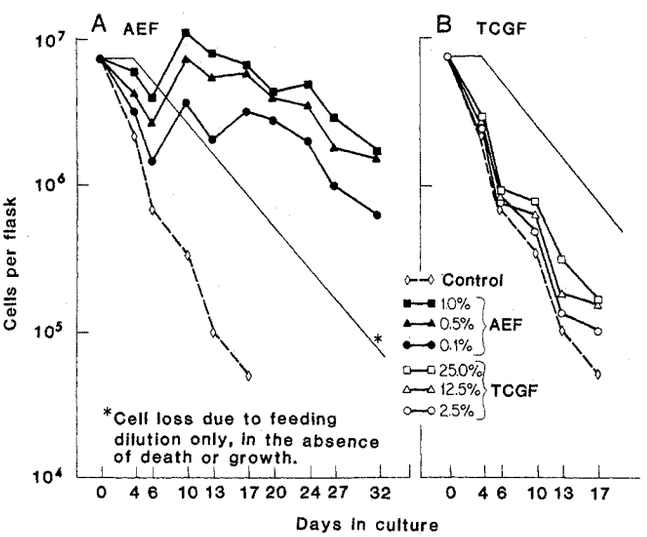

Fig. 1. Growth of CAF_1 bone marrow cells in the presence of AEF (A) and TCGF (B). Cultures were established with one inoculum of 7.5×10^6 fresh cells per flask with or without AEF or TCGF in the indicated concentrations. The AEF was prepared in serum-free conditions as described (*7*). The TCGF was derived from cultures of rat spleen cells (2×10^6 cells per milliliter) stimulated for 2 days with 5 μg of concanavalin A in RPMI-1640 plus 5 percent fetal calf serum. Several lower concentrations of TCGF had no significant effect on the cultures (data not shown). Shown are the actual numbers of nonadherent cells recovered from the cultures on days of feeding.

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