Emergence of Insulin Receptors upon Alloimmune T Cells in the Rat

J. HAROLD HELDERMAN and TERRY B. STROM

From the Immunology Laboratory, Renal Division, Department of Medicine, Peter Bent Brigham Hospital and Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT Insulin, as well as other ligands which increase intracellular guanosine 3',5'-cyclic monophosphate (cGMP), augments thymic-derived (T)-lymphocyte effector activity as revealed by alloimmune lymphocyte-mediated cytotoxicity. The observation that insulin binds only to monocytes among circulating nonimmune human mononuclear cells fostered reexamination of the mechanism by which insulin augments T-lymphocyte function. This report concerns a test of the hypothesis that the T cell is directly affected by insulin and that an insulin receptor emerges upon T lymphocytes consequent to immune activation. Spleens were removed from rats skin grafted across a major histocompatibility barrier. Lymphocytes were harvested from Ficoll-Hypaque density gradients and subsequently enriched for T cells by passage over one or two nylon wool columns. This population was composed of more than 95% T cells as assessed by surface marker techniques (Ig staining, erythrocyte antibody, and erythrocyte antibody complement rosetting, anti-T staining). There was no loss of augmentation of lymphocyte-mediated cytotoxicity induced by insulin, carbamycholine, and 8-bromo-cGMP in the purified cells when compared to unfractionated cells 7 days after transplantation. ^{125}I-insulin bound saturably to the allostimulated T-enriched lymphocytes with maximum binding at 12.8±0.2 pg and a dissociation constant at equilibrium of 1.3 nM. In contrast, insulin receptors were not present on nonimmune T-enriched cells or on T cells from animals that received syngeneic grafts. The affinity of the lymphocyte insulin receptor was similar to that of more conventional insulin-sensitive tissues e.g., liver, adipocyte. After 89% of T cells from spleens on day 7 were lysed with anti-thy 1.1 antibody and complement, the ability to measure specific insulin binding was lost. These data confirm a physiologic role for insulin in T-lymphocyte effector function and describe the emergence of insulin receptors concomitant with cell sensitivity to ligand. Such receptors may play a role in hormonal modulation of the immune response.

INTRODUCTION

The initial step in the action of most polypeptide hormones is a reversible interaction between free, unbound hormone and specific hormone receptor sites on the plasma membrane of a target cell (1–4). Such an interaction of ligand with receptor can be quantitatively analyzed utilizing mathematical constructs that define substrate-enzyme relationships (5). The characterization of the binding of insulin to its receptor has been extensively studied in a multiplicity of insulin-sensitive tissues including fat cells, fat cell membranes, liver cell membranes, and intact hepatocytes (4, 6–10). Insulin has been shown to bind to mononuclear lymphoid cells and to augment certain of their immunologic functions. The identity of the lymphoid cells capable of reversible insulin binding has been subject to controversy. Gavin and Archer and their respective co-workers could demonstrate that both bone marrow-derived (B) lymphoblastoid cell lines maintained in culture and Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) purified peripheral blood mononuclear leukocytes from normal blood donors had insulin receptors (11, 12). Krug et al. (13) could not detect such receptors on resting leukocytes collected after passage through nylon wool columns. The studies of Schwartz et al. (14) clearly demonstrated that insulin binding in any mixture of normal human peripheral blood mononuclear leukocytes is correlated to the presence of nylon wool-adherent monocyte macrophages and not to lymphocytes of either thymic-derived (T) or

A portion of this work was presented in abstract form in; 1976. *Clin. Res.* **24**: 337.

Dr. Strom is the recipient of a Research Career Development Award from the National Institute of Allergy and Infectious Diseases.

Received for publication 23 August 1976 and in revised form 25 October 1976.

B pedigrees. Consequently, cells partially purified by techniques which enrich specific cell populations, i.e. B or T cells, but do not delete monocytes, will demonstrate insulin binding. In contrast, cells collected by procedures which deplete macrophages, such as passage through a nylon wool column (15–18), will not bind insulin (14, 19).

Previously reported data have demonstrated that the ability of alloimmune splenic lymphocytes to lyse specifically ^{51}Cr-labeled donor cells is augmented by physiologic concentrations of insulin (20). The cell effecting lysis in the absence of insulin is a T lymphocyte (21–23). However, the absence of insulin receptors upon nonimmune human circulating T lymphocytes and the presence of monocyte macrophages and B cells in the insulin-treated spleen populations leave the mechanism of insulin-augmented lymphocyte-mediated cytotoxicity (LMC)[1] in doubt.

An intriguing report by Krug et al. (13) indicated that cells passed through nylon wool, presumably enriched for T lymphocytes and depleted of macrophages and B cells, develop an insulin receptor *de novo* after nonspecific mitogen stimulation by the plant lectin, concanavalin A. The study reported herein tests the hypothesis that resting T lymphocytes develop an insulin receptor after specific immune activation.

The cholinergic agonists, carbamylcholine and acetylcholine, which share with insulin the ability to increase intracellular guanosine 3′,5′-cyclic monophosphate (cGMP) (24–26) through activation of guanylate cyclase and exogenous 8-bromo-cGMP also enhance LMC (20, 27–30). It is possible that these agents do not influence LMC by directly altering the function of cytotoxic T lymphocytes since they also affect other cell types which might be involved (31–34). Therefore, the ability of insulin, cholinergic agonists, and 8-bromo-cGMP to augment LMC directly by affecting T cells was assessed utilizing B- and macrophage-depleted alloimmune-attacking cell populations.

METHODS

Cell preparation. Alloimmunization was accomplished by placing a skin graft from a (Lewis x Brown Norway) F_1 male rat onto a male Lewis rat. 7 days after grafting, spleens from the recipient animals were explanted after exsanguination. The cell suspension was produced by gently grinding the spleen through a 60-gauge steel mesh into RPMI-1640 media (Grand Island Biological Co., Grand Island, N. Y.), buffered with 0.5 volume % of Hepes and enriched with 5% (vol/vol) fetal calf serum (Grand Island Biological Co.). The suspension was washed once and layered on a Ficoll-Hypaque gradient (1.0956 *d*). Leukocytes were collected from the interface and washed twice. Cell suspensions from ungrafted Lewis rats or those Lewis rats given syngeneic skin grafts 7 days previously were prepared in the same manner. Aliquots of cells were utilized for the various studies detailed below.

Surface markers. Cells bearing receptors for the Fc portion of IgG were identified by formation of rosettes with ox erythrocytes complexed with IgG rabbit anti-ox erythrocyte antibodies (EA rosette) using a modification of the technique of Soulillou and co-workers (35). Cells bearing a receptor for complement (C) were detected by their ability to form rosettes with EAC indicator cells as previously described (35). Enumeration of lymphoid cells bearing surface Ig was performed by microscope inspection of cell suspensions incubated with fluorescein isothiocyanate-conjugated rabbit polyvalent anti-rat Ig (Cappel Laboratories, Inc., Downingtown, Pa.) (36). T lymphocytes were characterized by the microscope enumeration of cells with surface staining of fluorescein-labeled antibody directed against rat brain rendered specific for T cells by the method of Golub (37, 38). Macrophages were identified by morphologic criteria using acridine orange which was diluted with phosphate-buffered saline and added to 0.5×10^6 leukocytes to a final dilution of $1:10^6$ for 10 min at 24°C. The cells were examined after washing four times in buffered Hanks' solution. The robust cytoplasmic staining of macrophages allowed easy differentiation from lymphocytes (39, 40). We have previously determined that the cells identified as macrophages by this technique are identical to those bearing macrophage cytochemical characteristics utilizing pararosanilin solution.[2]

Insulin-binding assay. Spleen cells prepared as described above were washed twice in Hanks' solution, pH 7.4, containing 0.1% 5× recrystallized bovine serum albumin (Miles Laboratories, Inc., Elkhart, Ind.) and resuspended in this medium at 10×10^6 cells/ml. ^{125}I-insulin (supplied by New England Nuclear, Boston, Mass.) at a sp act of ~100 μCi/μg was prepared by the modification of Freychet et al. (6) of the Hunter and Greenwood method (41). The preparation is more than 90% monoiodinated (6) and biologically active (41, 42). A 200-μl aliquot of cells (2×10^6 cells/ml) was placed in siliconized 10 × 12-mm test tubes. To one set of triplicates 20 μl of porcine monocomponent insulin was added to a final concentration of 4 μg/ml; to another set, 20 μl of the buffer was added. After a 10-min incubation at 24°C, 20 μl of ^{125}-insulin (final concentrations ranging from 0 to 40 ng/ml) was added to replicate reaction mixtures and incubated for 45 min at 24°C. The incubations were terminated by a modification of the method of Gammeltoft and Gliemann (43, 44). To allow cells to sediment through the oil thus separating from the unbound label, the oil mixture was altered to be one part dinonyl phthalate: two parts dibutyl phthalate (vol/vol). Apparent binding affinity was estimated as the concentration giving half-maximal binding (apparent k_d or K_m) and calculated for each experiment by using a Hanes transformation of the binding data (45) for which the x-intercept provides the negative of the dissociation constant at equilibrium (k_d).

Depletion of T cells. An antiserum to the Thy 1.1 antigen present on AKR murine thymocytes with cross-reacts with rat splenic T cells was raised in C3H mice as described by Reif and Allen (46). Such antisera, in the presence of mouse lymphocyte-absorbed guinea pig complement, lysed around 55% of splenocytes and nearly 90% of thymocytes from Lewis rats. Complement alone did not lyse the Lewis lymphoid cells. Cell suspensions (100×10^6/0.5 ml) obtained from alloimmune Lewis animals and enriched for T cells were interacted with anti-Thy 1.1 antibody. The mixture was incubated at 4°C for 30 min with occasional gentle shaking,

[1] *Abbreviations used in this paper*: C, complement; EA, erythrocyte antibody rosette; k_d, dissociation constant at equilibrium; LMC, lymphocyte-mediated cytotoxicity.

[2] Strom, T. B. et al., unpublished observations.

TABLE I
Cell Marker Identification of Ficoll-Hypaque, Nylon Wool-Sensitized Lymphocytes

Cell markers	%
Ig Staining	3
EA Rosette	5
EAC Rosette	4
Anti-T cell	95
Macrophages (Acridine orange)	0

washed in minimal essential medium and resuspended in 0.5 ml of minimal essential medium containing 10% fetal calf serum. 1 ml of the absorbed guinea pig complement previously diluted 1:1 with minimal essential medium was added. The mixture was further incubated for 40 min at 37°C in a shaking water bath. The mixture was washed twice in medium by centrifugation at 150 *g* for 15 min. The percentage of cells killed was calculated by counting the viable cells after treatment (i.e., cells that exclude eosin or trypan blue). The cells were then washed in Hanks'-0.1% bovine serum albumin, pH 7.4, adjusted to the initial volume of medium and assayed for specific ^{125}I-insulin binding as described. Cells prepared from the same engrafted animals but not treated with this anti-T-cell antibody served as a positive control.

Nylon wool separation. Host lymphoid populations were separated into adherent and nonadherent cell fractions by nylon wool filtration using the method of Handwerger and Schwartz (17). The nonadherent cells were collected by filtration through a nylon wool column packed into a 20-ml syringe. Macrophages were also deleted from splenic cell suspensions by their adherence to plastic petri dishes.

Quantitation of LMC. The cytotoxic action of alloimmune splenocytes on thymocytes bearing alloantigens to which they are sensitized (LMC) was determined by a previously described modification (27, 28) of the technique of Brunner et al. (47). Pharmacologic agents were diluted in tissue culture medium just before use and were interacted with the attacking lymphocytes for time intervals varying from 0 to 10 min at room temperature before the introduction of the target cells. The pharmacologic agents did not injure the attacking or target cells as determined in ^{51}Cr-release studies.

The percent specific lysis was determined as follows:

$$\left[\frac{\text{Experimental cpm} - \text{control cpm}}{\text{Freeze thaw cpm} - \text{control cpm}}\right] \times 100.$$

The percent augmentation of specific lysis observed with pharmacologic treatment was determined as follows:

$$\left(\left[\%\,\frac{\text{specific lysis in treated mixture}}{\text{specific lysis in untreated mixture}}\right] - 1\right) 100.$$

TABLE II
*Pharmacologic Augmentation of LMC after 7-Day Skin Allografts**

Agent, concentration	n‡	Unfractionated spleen	Nylon wool effluent	Petri dish nonadherent
		%	%	%
Insulin,§				
0.1–1 nM	7	46±4*	48±4	46±6
8-bromo-cGMP,‖				
5 μM	6	42±5	46±6	46±5
Carbamylcholine,¶				
1–10 pM	6	37±5	39±5	36±5

* Mean±SEM (percent augmentation).
‡ Number of experiments.
§ LMC without insulin 38.5±8.0% (specific chromium release).
‖ LMC without 8-bromo-cGMP 41.2±7.4% (specific chromium release).
¶ LMC without carbamylcholine 42.3% (specific chromium release).

RESULTS

Cell surface markers. Cell surface marker studies on the cell populations utilized in this study are summarized in Table I. The passage of Ficoll-Hypaque-harvested cells through nylon wool twice produced a population composed of 95% T lymphocytes and devoid of cells with the appearance of macrophages as assessed by acridine orange staining. Surface Ig-positive lymphocytes constituted 3% of the total cells. The number of cells that form EA rosettes among nylon wool-filtered cells is small (4–6%), and closely approximated the number of B cells as determined by surface Ig staining. Polymorphs were easily excluded from EA scoring because of their easily identifiable nuclear morphology. Cells demonstrating a surface receptor for complement formed a small percentage of the nylon wool-filtered cells. These cells had morphologic characteristics of lymphocytes and were present in numbers that again approximated closely the numbers of the Ig-positive B cells.

Lymphocyte-mediated cytotoxicity. As previously reported, increased LMC was discerned when the sensitized attacking cells were preincubated with optimal concentrations of the pharmacologic agents, insulin (0.1–1 nM), carbamylcholine (1–10 pM), and 8-bromo-cGMP (5 μM) for 1–8 min before the introduction of target cells. The maximal pharmacologic augmentation of LMC produced in each cell pool was compared using Ficoll-Hypaque-purified populations and an aliquot of cells from the same pool passed through nylon wool. It is readily apparent that nylon wool filtration did not alter the degree of pharmacologically augmented cell lysis (Table II). Elimination of cells adherent to plastic petri dishes also failed to alter the magnitude of pharmacologically augmented LMC (Table II).

Insulin binding. Insulin does not bind to the membranes of nylon wool-filtered lymphocytes harvested

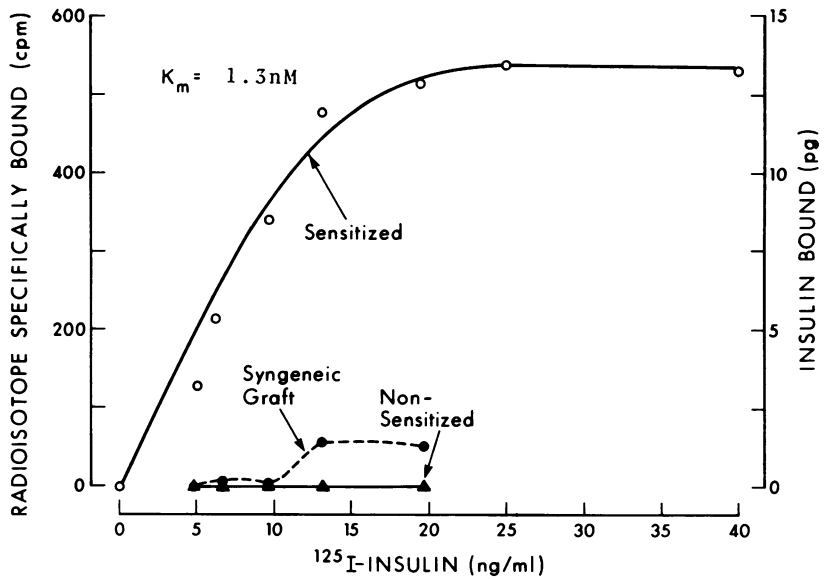

FIGURE 1 Saturable binding of ^{125}I-insulin to allostimulated T cells. There are no discernible insulin receptors on nonimmune T cells or on T cells from animals which had received syngeneic skin grafts.

from unimmunized Lewis animals (Fig. 1). Nonadherent lymphocytes harvested 7 days after syngeneic skin grafting also failed to bind insulin.

In contrast, specific binding of insulin to nylon wool, effluent lymphocytes harvested from Lewis rats 7 days after allogeneic skin grafting is demonstrable. This binding exhibits saturable kinetics (Fig. 1). The half-maximal saturation, an index of apparent k_d, occurred at a mean of 1.3 nM which is almost precisely the same affinity for binding to nylon nonadherent lymphocytes (T lymphocytes by inference) found by Krug and her co-workers (1 nM) (13). If the assumption is made that all T lymphocytes bind insulin equally on their membranes, one can calculate from the maximum binding of 12.8±0.2 pg (1.3×10^9 molecules) (mean±1 SEM) to the 2×10^6 cells present in each assay that there are 671±82 molecules of peptide bound per cell; however, it is unlikely that all of the T lymphocytes in the cell suspension bind insulin. A Hanes transformation of the data (Fig. 2) gives 0.9 nM for the k_d of the receptor lending support to the presence of a binding site that efficiently binds nanomolar concentrations of insulin.

Deletion of T cells. The interaction of anti-Thy 1.1 antibody (anti-T-cell antibody) in the presence of complement lysed 89% of the T-enriched alloimmune splenic lymphocytes that passed through a nylon wool column. The anti-Thy 1.1 and C-treated nylon wool-filtered alloimmune cells did not bind insulin whereas an aliquot of cells from the same cell pool filtered through nylon wool and not treated with anti-Thy 1.1 + C bore demonstrable insulin-binding sites evidenced by binding with an apparent k_d of 0.83 nM (Fig. 3). Treatment with guinea pig complement alone did not influence binding.

DISCUSSION

Hormones and neurotransmitters are informational molecules. These ligands react only with target cells bearing specific hormonal recognition units. The recognition units or receptors bind hormone with high affinity and rigorous specificity. A variety of ligands that are known to influence the behavior of receptor-bearing target cells, presumably by activation of adenylate cyclase or guanylate cyclase, with formation of intracellular cAMP or cGMP, alter the ability of

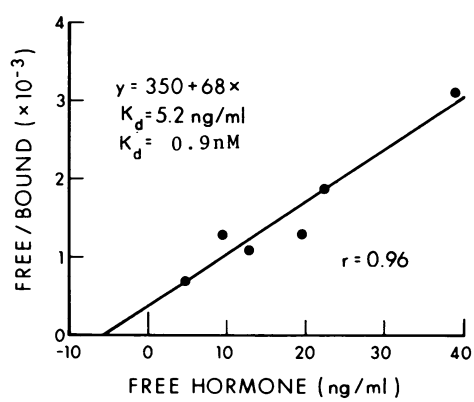

FIGURE 2 A Hanes transformation of ^{125}I-insulin binding to sensitized T cells in a representative experiment.

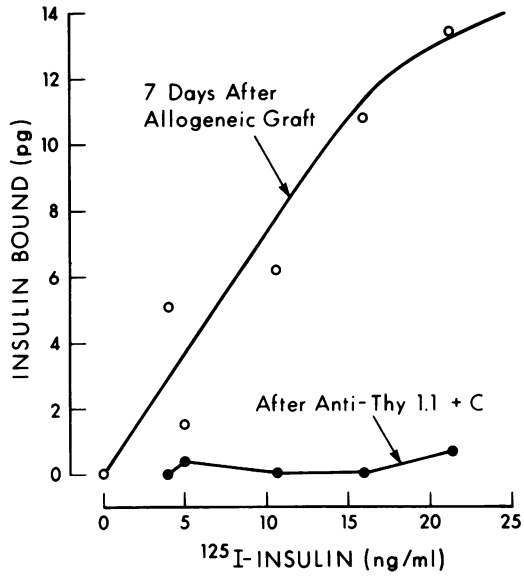

FIGURE 3 Abrogation of ^{125}I-insulin binding on stimulated T cells by anti-T-cell antibody and complement. Untreated, sensitized T cells from the same animals give a characteristic binding curve with an apparent k_d of 1.4 nM.

alloimmune rat splenic lymphocytes to destroy cells bearing the immunizing alloantigens. Activators of adenylate cyclase inhibit cytotoxicity (27, 28, 30, 48, 49) whereas ligands reported to activate guanylate cyclase such as cholinergic agonists (28, 29) and insulin (20) augment LMC.

LMC is mounted by alloimmune cytotoxic T lymphocytes; however, nonimmune human circulating blood T lymphocytes do not bear a receptor for insulin (14). The data described herein demonstrate that in vivo alloimmunization results in the appearance in rat T-splenic lymphocyte populations of receptors with the ability to bind insulin with saturation kinetics. T lymphocytes in the resting state or after syngeneic skin grafting do not bind insulin (Fig. 1). The identification of insulin-binding cells as alloimmune lymphocytes is crucial to the reconciliation of the view that resting mononuclear leukocytes collected on Ficoll-Hypaque gradients have insulin receptors (11, 12), whereas T-enriched nonimmune cells percolated through columns packed with nylon wool have none (13, 14). Since Schwartz et al. (14) identified the insulin-binding cells in peripheral blood as nylon wool-adherent macrophage monocytes, pains were taken to eliminate macrophage monocytes by the techniques employed by these workers (Table I) and to identify binding cells as T in character. Since control cells without binding and sensitized cells with this property were prepared identically, the presence of a small percentage of undetected macrophages within these cell preparations would not explain the appearance of insulin-binding cells unless allografting allowed more macrophages to escape entrapment within the nylon wool. Most importantly, an antiserum directed against the murine Thy 1.1 antigen which cross-reacts with rat splenic T lymphocytes (46) (but not macrophages) in the presence of complement lysed 89% of the T-enriched alloimmune spleen cells and totally abolished their ability to bind insulin.

Although previous data demonstrated that the depletion of B cells by anti-immunoglobulin and complement did not alter pharmacologically augmented LMC (20, 28), the possibility that macrophages are crucial to augmented lysis had not been assessed. The data shown in Table II demonstrate that T-enriched, B-depleted, and macrophage-free nylon wool-filtered populations are equally responsive to the immunopotentiating properties of 8-bromo-cGMP, carbamylcholine, and insulin. Similarly, the elimination of cells adherent to petri dishes did not diminish the magnitude of pharmacologically augmented LMC (Table II). Since these agents do not cause the development of cytotoxicity in nonimmune populations, it seems certain that insulin, carbamylcholine, and 8-bromo-cGMP exert their influence upon activated T lymphocytes and probably directly upon cytotoxic T lymphocytes.

The appearance or unmasking of the receptor could be a marker of lymphocyte activation without playing an inherent role in the events of immune function. The fact that lymphoblasts from leukemia patients and normal lymphocytes transformed by the plant lectin concanavalin A (13) develop these receptors support the view that the appearance of an insulin receptor occurs as a consequence of T-cell activation. However, the fact that insulin can augment at least one immune function of sensitized lymphocytes, i.e. LMC, suggests a substantive physiologic role for insulin and its receptor in modulating the character and vigor of imunological processes. Alloimmune T cells develop insulin receptors as they develop the ability to attack and destroy allogeneic target cells to which they have been immunized with the result that immune "killer" function can be augmented by the ligand, insulin (20). Because of the requirement that the attacking cells be exposed to insulin for a precise time interval before introduction of target cells (20), it is unlikely that the peptide functions in a nonspecific manner. This time-dependent, optimal dose-dependent enhancement of T-cell function (as measured by the LMC) suggests a functional link between insulin binding and immune response.

ACKNOWLEDGMENTS

We would like to thank Dr. Ronald Chance of the Eli Lilly Company in Indianapolis for supplying the monocom-

ponent insulin used in this study. Thanks are also due to Jean Paradysz and Ruth Kostick for skillful technical assistance, to Lynne Magnussen for careful preparation of the manuscript, and to Dr. M. D. Hollenberg and Dr. C. B. Carpenter for advice and encouragement.

This work was supported in part by NIH grant CA-16937 and a grant from the Leukemia Research Foundation, Inc.

REFERENCES

1. Pastan, I., J. Roth, and V. Macchia. 1966. Binding of hormone to tissue: the first step in polypeptide hormone action. *Proc. Natl. Acad. Sci. U. S. A.* **56**: 1802–1809.
2. Cuatrecasas, P. 1969. Interaction of insulin with the cell membrane: the primary action of insulin. *Proc. Natl. Acad. Sci. U. S. A.* **63**: 450–457.
3. Lefkowitz, T., J. Roth, W. Pricer, and I. Pastan. 1970. ACTH receptors in the adrenal: specific binding of ACTH-^{125}I and its relation to adenyl cyclase. *Proc. Natl. Acad. Sci. U. S. A.* **65**: 745–752.
4. Freychet, P., J. Roth, and D. M. Neville, Jr. 1971. Insulin receptors in the liver: specific binding of [^{125}I]insulin to the plasma membrane and its relation to insulin bioactivity. *Proc. Natl. Acad. Sci. U. S. A.* **68**: 1833–1837.
5. Boeynaems, J. M., and J. E. Dumont. 1975. Quantitative analysis of the binding of ligands to their receptors. *J. Cyclic Nucleotide Res.* **1**: 123–142.
6. Freychet, P., J. Roth, and D. M. Neville, Jr., 1971. Monoiodoinsulin: demonstration of its biological activity of binding to fat cells and liver membranes. *Biochem. Biophys. Res. Commun.* **43**: 400–408.
7. House, P. D. R., and M. J. Weidemann. 1971. Characterization of [^{125}I]-insulin binding plasma membrane fraction from rat liver. *Biochem. Biophys. Res. Commun.* **41**: 541–548.
8. Cuatrecasas, P. 1971. Unmasking of insulin receptors in fat cells and fat cell membranes. Perturbation of membrane lipids. *J. Biol. Chem.* **246**: 6532–6542.
9. Cuatrecasas, P., B. Desbuquois, and F. Krug. 1971. Insulin-receptor interactions in liver cell membranes. *Biochem. Biophys. Res. Commun.* **44**: 333–339.
10. Cuatrecasas, P. 1972. Affinity chromatography and purification of the insulin receptor of liver cell membranes. *Proc. Natl. Acad. Sci. U. S. A.* **69**: 1277–1281.
11. Gavin, J. R., III, J. Roth, P. Jen, and P. Freychet. 1972. Insulin receptors in human circulating cells and fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **69**: 747–751.
12. Archer, J. A., P. Gorden, J. R. Gavin, III, M. A. Lesniak, and J. Roth. 1973. Insulin receptors in human circulating lymphocytes: application to the study of insulin resistance in man. *J. Clin. Endocrinol. Metab* **36**: 627–633.
13. Krug, U., F. Krug, and P. Cuatrecasas. 1972. Emergence of insulin receptors on human lymphocytes during *in vitro* transformation. *Proc. Natl. Acad. Sci. U. S. A.* **69**: 2604–2608.
14. Schwartz, R. H., A. R. Bianco, B. S. Handwerger, and C. R. Kahn. 1975. Demonstration that monocytes rather than lymphocytes are the insulin-binding cells in preparation of human peripheral blood mononuclear leukocyte. Implications for studies of insulin-resistant states in man. *Proc. Natl. Acad. Sci. U. S. A.* **72**: 474–478.
15. Greaves, M. F., and G. Brown. 1974. Purification of human T and B lymphocytes. *J. Immunol.* **112**: 420–423.
16. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3**: 645–649.
17. Handwerger, B. S., and R. H. Schwartz. 1974. Separation of murine lymphoid cells using nylon wool columns. Recovery of the B cell-enriched population. *Transplantation (Baltimore).* **18**: 544–547.
18. Oppenheim, J. J., B. G. Leventhal, and E. M. Hersh. 1968. The transformation of column-purified lymphocytes with nonspecific and specific antigenic stimuli. *J. Immunol.* **101**: 262–270.
19. Olefsky, J., and G. M. Reavan. 1974. The human lymphocyte: A model for the study of insulin-receptor interaction. *J. Clin. Endocrinol. Metab.* **38**: 554–560.
20. Strom, T. B., R. A. Bear, and C. B. Carpenter. 1975. Insulin-induced augmentation of lymphocyte-mediated cytotoxicity. *Science (Wash. D. C.).* **187**: 1206–1208.
21. Golstein, P., H. Wigzell, H. Blomgren, and E. A. J. Svedmyr. 1972. Cells mediating specific in vitro cytotoxicity. II. Probable autonomy of thymus-processed lymphocytes (T cells) for the killing of allogeneic target cells. *J. Exp. Med.* **135**: 890–906.
22. Cerottini, J.-C., A. A. Nordin, and K. T. Brunner. 1970. *In vitro* cytotoxic activity of thymus cells sensitized to alloantigens. *Nature (Lond.).* **227**: 72–73.
23. Hersey, P. 1973. Thymus-dependent cytotoxic lymphocytes in the rat. *Eur. J. Immunol.* **3**: 748–754.
24. George, W. J., J. B. Polson, A. G. O'Toole, and N. D. Goldberg. 1970. Elevation of guanosine 3′,5′-cyclic phosphate in rat heart after perfusion with acetylcholine. *Proc. Natl. Acad. Sci. U. S. A.* **66**: 398–403.
25. Goldberg, N. D., M. K. Haddox, R. Estensen, J. G. White, C. Lopez, and J. W. Hadden. 1974. Evidence of a dualism between cyclic GMP and cyclic AMP in the regulation of cell proliferation and other cellular processes. *In* Cyclic AMP, Cell Growth, and the Immune Response. Braun, W., L. M. Lichtenstein, C. W. Parker, editors. Springer-Verlag New York Inc., New York. 247–262.
26. Illiano, G., G. P. E. Tell, M. I. Siegel, and P. Cuatrecasas. 1973. Guanosine 3′:5′-cyclic monophosphate and the action of insulin and acetylcholine. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 2443–2447.
27. Strom, T. B., A. Deisseroth, J. Morganroth, C. B. Carpenter, and J. P. Merrill. 1972. Alteration of the cytotoxic action of sensitized lymphocytes by cholinergic agents and activators of adenylate cyclase. *Proc. Natl. Acad. Sci. U. S. A.* **69**: 2995–2999.
28. Strom, T. B., C. B. Carpenter, M. R. Garovoy, K. F. Austen, J. P. Merrill, and M. Kaliner. 1973. The modulating influence of the cyclic nucleotides on lymphocyte-mediated cytotoxicity. *J. Exp. Med.* **138**: 381–388.
29. Strom, T. B., A. J. Sytkowski, C. B. Carpenter, and J. P. Merrill. 1974. Cholinergic augmentation of lymphocyte-mediated cytotoxicity. A study of the cholinergic receptor of cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **71**: 1330–1333.
30. Garovoy, M. R., E. J. Ball, R. E. Lordon, and A. L. Thompson. 1975. Modulation of cytolytic ability of human T and K-lymphocytes in direct and antibody dependent lymphocyte mediated cytotoxicity by agents affecting intracellular levels of cAMP and cGMP. *Clin. Res.* **23**: 290A. (Abstr.)
31. Weinstein, Y., S. Segal, and K. L. Melmon. 1975. Specific mitogenic activity of 8-Br-guanosine 3′,5′-monophosphate (Br-cyclic GMP) on B lymphocytes. *J. Immunol.* **115**: 112–117.

32. Diamanstein, T., and A. Ulmer. 1975. Effect of cyclic nucleotides on DNA synthesis in mouse lymphoid cells. *Immunol. Commun.* **4**: 51–62.
33. Diamanstein, T., and A. Ulmer. 1975. Stimulation by cyclic GMP of lymphocytes mediated by soluble factor released from adherent cells. *Nature (Lond.).* **256**: 418–419.
34. Schmidtke, J. R., and S. Hatfield. 1976. Activation of purified human thymus-derived (T) cells by mitogens. II. Monocyte-macrophage potentiation of mitogen-induced DNA synthesis. *J. Immunol.* **116**: 357–362.
35. Soulillou, J-P., C. B. Carpenter, A. J. F. d'Apice, and T. B. Strom. 1976. The role of nonclassical, Fc-receptor-associated, Ag-B antigens (Ia) in rat allograft enhancement. *J. Exp. Med.* **43**: 405–421.
36. Raff, M. C., M. Sternberg, and R. B. Taylor. 1970. Immunoglobulin determinants on the surface of mouse lymphoid cells. *Nature (Lond.)* **225**: 553–554.
37. Golub, E. S. 1971. Brain-associated theta antigen: reactivity of rabbit anti-mouse brain with mouse lymphoid cells. *Cell. Immunol.* **2**: 353–361.
38. Golub, E. S. 1972. The distribution of brain associated θ antigens cross-reactive with mouse in the brain of other species. *J. Immunol.* **109**: 168–170.
39. Melamed, M. R., L. R. Adams, A. Zimring, J. G. Murnick, and K. Mayer. 1972. Preliminary evaluation of acridine orange as a vital stain for automated differential leucocyte counts. *Am. J. Clin. Pathol.* **57**: 95–102.
40. Strom, T. B., N. L. Tilney, C. B. Carpenter, and G. J. Busch. 1975. Identity and cytotoxic capacity of cells infiltrating renal allografts. *N. Engl. J. Med.* **292**: 1257–1263.
41. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature (Lond.).* **194**: 495–496.
42. Sodoyez, J. C., M. M. Goff, and E. R. Arquilla. 1974. Preparation of carrier free ^{125}I monoiodoinsulin and biological properties of monoiodoinsulin. *Diabetes.* **23** (Suppl. 1): 353. (Abstr.)
43. Gammeltoft, S., and J. Gliemann. 1973. Binding and degradation of ^{125}I-labelled insulin by isolated rat fat cells. *Biochem. Biophys. Acta.* **320**: 16–32.
44. Gliemann, J., K. Østerlind, J. Vinten, and S. Gammeltoft. 1972. A procedure for measurement of distribution spaces in isolated fat cells. *Biochem. Biophys. Acta.* **286**: 1–9.
45. Hanes, C. S. 1932. Studies on plant amylases. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. *Biochem. J.* **26**: 1406–1421.
46. Reif, A. E., and J. M. V. Allen. 1964. The AKR thymic antigen and its distribution in leukemias and nervous tissues. *J. Exp. Med.* **120**: 413–433.
47. Brunner, J. Mauel, J-C. Cerottini, and B. Chapuis. 1968. Quantitative assay of the lytic action of immune lymphoid cells on ^{51}-Cr-labelled allogeneic target cells *in vitro*: inhibition by isoantibody and by drugs. *Immunology.* **14**: 181–196.
48. Henney, C. S., and L. M. Lichtenstein. 1971. The role of cyclic AMP in the cytolytic activity of lymphocytes. *J. Immunol.* **107**: 610–612.
49. Henney, C. S., H. R. Bourne, and L. M. Lichtenstein. 1972. The role of cyclic 3′,5′ adenosine monophosphate in the specific cytolytic activity of lymphocytes. *J. Immunol.* **108**: 1526–1534.