Immunobiological Consequence of Regulation of Insulin Receptor on Alloactivated Lymphocytes in Normal and Obese Subjects

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Acute manipulations of insulin in vivo regulate the display of insulin receptors induced on activated T lymphocytes after presentation of alloantigen. This study explored the immunobiological consequences of regulation of insulin-receptor display by acute manipulations of insulin achieved during the hyperinsulinemic-euglycemic clamp in healthy normal individuals and obese subjects. T lymphocytes were isolated at 0, 1, and 4 h of hyperinsulinemia from seven normal volunteers and seven obese individuals and studied for their capacity to *1*) synthesize a complement of insulin receptors on cell membrane, *2*) respond to alloantigen in the mixed-lymphocyte culture (an immunologic activity unrelated to manipulations in insulin concentrations in complete medium), and *3*) respond to the lymphocyte-mediated cytotoxicity reaction (an immunologic activity known to be modulated by insulin). In the face of a reduction in receptor numbers to 25% of baseline in normal individuals, alloreactivity in the mixed-lymphocyte culture was not affected (95 ± 9% of time 0 after 4 h of hyperinsulinemia), whereas lymphocyte-mediated cytotoxicity fell from 14 ± 4 at time 0 to 2 ± 2% sp Cr release ($P < 0.02$). Hyperinsulinemia achieved by the clamp in seven obese subjects did not alter the synthesis of insulin receptors on cell membrane after presentation of alloantigen. In the absence of further reduction of insulin-receptor membrane display, neither the mixed-lymphocyte culture nor lymphocyte-mediated cytotoxicity reaction was affected. It is concluded that those immunologic activities of lymphocytes that can be modulated by insulin are affected by regulation of insulin-receptor display on activated lymphocytes. Therefore, receptor regulation is not effete but carries significant immunologic consequence. This study supports the hypothesis that insulin-directed immunologic activity is regulated at the site of receptor display rather than at the locus of regulation of the ligand itself. *Diabetes* 40:364–70, 1991

Insulin, like numerous hormones, neurotransmitters, and autacoids, is an immunobiologically relevant molecule that participates in the regulation of immune responses to antigen and lectin (1). This hormone exerts its biological effect on immunocompetent cells through the intercession of a binding event with a specific receptor on the cell surface that is a unique synthetic product of the activation process during cell response to nominal antigen (2,3). For example, insulin enhances the ability to generate cytotoxic lymphocytes in response to alloantigen (1,4). Supraphysiological but not physiological concentrations of insulin can augment blastic transformation initiated by alloantigen (5–7). We have shown previously that the presence of an insulin receptor on activated lymphocytes permits that ligand to *1*) maintain the activated state of the lymphocyte when submaximal antigen stimuli are tendered, *2*) quantitatively regulate the amount of cytotoxic T-lymphocyte function in the presence of sensitizing target cells, and *3*) regulate substrate transport for intermediary metabolism on both T and B lymphocytes (1,2,8–10). Because the immunocompetent cell does not participate in the feedback regulation of plasma insulin, it must adjust the nature of the insulin-directed signal by altering the display of the ligand receptor. Thus, it seems reasonable to propose that insulin-receptor regulation on the activated T lymphocyte is crucial for the regulation of the insulin immunobiological response.

We have been interested in describing the mechanisms by which the insulin receptor is regulated on these immunocompetent cells and have revealed the presence of an inverse relationship between the concentration of plasma insulin, set chronically by disordered carbohydrate metab-

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olism or acutely raised by the glucose-clamp technique, and the ultimate display of receptors as stimulated in tissue culture by antigen (11–13). This study was designed to specifically attempt to link fine regulation of the display of the insulin receptor on activated T lymphocytes with biological consequence. We explored the immunobiological consequences of regulation of insulin-receptor display by acute manipulation of plasma insulin in normal volunteers and obese subjects, whose basal display of the insulin receptor on activated lymphocytes is disrupted.

RESEARCH DESIGN AND METHODS

Two groups of subjects were chosen for analysis (Table 1). The first study group was composed of seven normal-weight young subjects with normal parameters of plasma insulin and plasma glucose who were known to exhibit ligand-directed regulation of the insulin receptor on activated thymus-derived lymphocytes. They had normal glucose tolerance, no history of diabetes mellitus in first-degree relatives, and normal glycosylated hemoglobin. The normal group permitted analysis of the immunobiological consequences of acute perturbations in plasma insulin that might parallel fine regulation of the insulin receptor on activated T lymphocytes previously reported (13). A second group was chosen to be at least 50% above ideal body weight (Metropolitan Life tables) and to have chronically elevated plasma insulin (126 ± 6 pM) in the face of normal blood plasma glucose (5.4 ± 0.6 mM) and glycosylated hemoglobin. Previous studies revealed that this obese group would have a basal diminution in the number of insulin receptors displayed on T lymphocytes, the synthesis of which is stimulated by antigen or lectin (12). This group permitted analysis of *1*) the means by which acute perturbation in plasma insulin might lead to defects in the fine regulation of the T-lymphocyte insulin receptor during chronic hyperinsulinemia and *2*) the immunobiological consequence of the potential disruption in fine regulation of the receptor.

This study was conducted according to the principles of the Declaration of Helsinki and was approved and monitored by the Institutional Review Board of the University of Texas Southwestern Medical Center.

Clamp protocol. The model for study was the hyperinsulinemic-euglycemic clamp technique described originally by DeFronzo et al. (14), which we previously used to effect acute perturbations in the plasma insulin leading to fine regulation of the display of the insulin receptor on activated T lymphocytes stimulated by alloantigen in tissue culture (13,15). The assumptions and mathematical algorithms that underlie the performance of the euglycemic clamp have been described and have been used by us in a series of previous studies (13,15–17). In brief, subjects, after providing informed and free consent under the scrutiny of the Institutional Review Board of the University of Texas Southwestern Medical Center, were studied in the Clinical Research Center after an overnight fast. Plasma glucose concentrations were maintained near or at the euglycemic goal (coefficient of variation ≤5% for an acceptable study) by the use of a negative feedback algorithm with assays of "arterialized" blood glucose measured every 2.5 min for the first 30 min of study and every 5 min for the remainder of the 240-min study. Blood for measurements of immunoreactive insulin was obtained every 20 min for the 1st h and every 15 min for the duration of the study. For the immune-function studies, heparinized blood samples were obtained with sterile technique at 0, 1, and 4 h of hyperinsulinemia and reserved for isolation of T lymphocytes for tissue culture and ultimate analysis of function and receptor display.

Mixed-lymphocyte culture (MLC) and lymphocyte-mediated cytotoxicity (LMC). Mononuclear cells were isolated from sterile heparinized blood removed during the clamp as described above. Each sample was diluted 1:3 with Hank's balanced salt solution and layered over a Ficoll-Hypaque density gradient as previously described (4). The mononuclear cells obtained at the interface of the density gradient were washed extensively and reserved for two studies of immune function, the MLC, an immunologic assay known to be independent of regulation by insulin (8,18), and LMC, an assay known to be dependent on insulin (1,4).

To analyze the impact of receptor regulation created by acute perturbation in plasma insulin on an assay of immune function not modulated by insulin, MLCs were established. To establish the cultures, 100 μl of the mononuclear cells at 10^6 cells/ml "enriched medium" (RPMI-1640 enriched with 5% fetal calf serum containing 0.24 pM beef insulin and buffered with 10 mM HEPES) were placed in wells of a 96-well microculture plate (Costar, Cambridge, MA) along with an equal volume and number of mononuclear cells obtained from a panel of volunteers whose cells were known to be HLA-DR disparate from the clamped individual to provide alloantigen stimulation. Culture was effected for 6 days at 37°C in 95% air/5% CO_2. On the 6th day, cultures were pulsed with 1 μCi/well [^{3}H]thymidine for 4 h, followed by harvest on glass-fiber filters with a Mash II harvesting system (Microbiology Associates, Bethesda, MD). Incorporation of [^{3}H]thymidine into responder cells was evaluated with analysis of β-radiation in a scintillation counter (Beckman, Palo Alto, CA).

To analyze the impact of receptor regulation created by

TABLE 1
Patient characteristics before and after 4-h hyperinsulinemic-euglycemic clamp

					During clamp	
	Age (yr)	Obesity index	Fasting plasma glucose (mM)	Fasting IRI (pM)	Plasma glucose (mM)	IRI (pM)
Normal	27.0 ± 1.1	1.1 ± 0.1	5.17 ± 0.13	46.8 ± 5.4	5.11 ± 0.02	602.4 ± 13.2
Obese	31.3 ± 2.3	1.6 ± 0.1	5.40 ± 0.05	123.6 ± 7.2	5.36 ± 0.01	666.7 ± 19.2

Values are means ± SE for $n = 7$ in each group. IRI, immunoreactive insulin. Obesity index, ratio of weight to ideal weight by Metropolitan Life tables.

acute perturbation in plasma insulin on an assay of immune function modulated by insulin, LMC assays were performed. Mononuclear cells isolated from blood obtained during 4 h of hyperinsulinemia created by the glucose clamp were planted in MLCs for alloantigenic stimulation of killer T lymphocytes. Mononuclear cells (10 ml) from clamped patients at 10^6 cells/ml were incubated in microculture flasks (Costar) along with an equal number of cells from the stimulator partner effecting an alloantigenic stimulus. Culture was continued for 6 days at 37°C in an atmosphere of 95% air/5% CO_2. On the 6th culture day, fresh mononuclear cells were removed from the stimulator subject responsible for the MLC and were labeled with [^{51}Cr]sodium chromate (200–500 μCi; ICN, Irvine, CA) as previously described to serve as the target cells bearing the sensitizing antigen (1,4). Potential cytotoxic cells were recovered from the MLC after centrifugation of the macroculture at 3000 rpm (Beckman) and resuspension at 10^6 cells/ml. Potential killer lymphocytes (100 μl) were placed in 10 × 12-mm test tubes and incubated with an equal volume of Cr-labeled targets at 10^4/ml (killer-target ratio 100:1) in enriched medium with serum containing a small concentration of beef insulin (<0.24 pM) that was identical for cultures from all patients and test conditions for 30 min at 37°C. The in vitro concentration of plasma insulin created by glucose clamping, from which the cells were derived, was the variable of interest and not the insulin concentration in the in vitro cultures, which was fixed for all cells. The tubes were gently centrifuged at 50 × *g* in a Beckman table-top centrifuge followed by an additional 3.5-h incubation at 37°C. The cultures were then spun at 500 × *g* for 10 min. Released tracer was recovered by removal of 100 μl of supernatant followed by analysis of γ-radiation in a γ-counter. Spontaneous release was ascertained by incubating 100 μl of targets at 10^4/ml with 100 μl of nonsensitized filler mononuclear cells in enriched medium handled identically to the active killer T lymphocytes. Maximum release was measured by incubating 100 μl of targets (10^4/ml) with 100 μl 0.1 N HCl carried through the identical LMC process as the active condition.

Percent specific Cr release was calculated with the formula

$$\left(\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}}\right) \times 100$$

By convention, a positive LMC is taken as ≥2% sp Cr release.

Insulin-binding assay. To ascertain that acute perturbation of plasma insulin exerted the expected effect on the fine regulation of T-lymphocyte insulin-receptor display on alloactivated lymphocytes from normal subjects, cells removed at 0, 1, and 4 h of the euglycemic clamp from this study group and placed into MLCs as described above were reserved for insulin-receptor binding analysis. The insulin-binding assay also permitted comments concerning the possibility that fine regulation of insulin-receptor display on activated lymphocytes during chronic hyperinsulinemia in obese subjects might be defective.

The mononuclear cells from the MLC were further separated into T-lymphocyte–enriched fractions by the double nylon-wool technique previously described and validated, which routinely provides fractions containing >95% T lymphocytes as adjudged by surface markers with monoclonal antibody technology and fluorescence-activated cell analysis (4,9,13,19). These T lymphocytes were subjected to a radioligand-binding assay for the insulin receptor that has been reported previously and extensively characterized and validated (2–4,7,11,13,15,19). The nature of nonspecific binding, the shape and form of the Scatchard relationship, and the success of separation of bound from free radiolabeled insulin receptor have also been described previously by us with this model system. Specific binding of [^{125}I]iodoinsulin to activated lymphocytes obtained at the time of harvest of the MLC on day 6 was measured under equilibrium binding conditions after the provision of trace iodoinsulin at a final concentration of 10 ng/ml as previously described for the measurement of receptor characteristics after harvest of cells from the various test cultures (3). Specific binding was determined as the difference between total binding of trace ligand and nonspecific binding determined in the presence of 100-fold excess of cold ligand. The use of a single binding point parallels our previous studies and was necessitated by the large amount of blood needed to accomplish the two immunobiological studies and the binding studies. To ascertain the nature of potential diminution in radioligand binding effected by acute perturbation of insulin during the clamp, enough blood was obtained in two additional studies from each of the study groups at time 0 and 4 h of clamp to construct complete association binding isotherms, the data of which were subjected to standard Scatchard analysis.

Other methods. To ascertain the effect of clamped conditions on the number of cytotoxic/suppressor T lymphocytes present in the study subjects, five additional 4-h hyperinsulinemic-euglycemic clamp studies were performed in normal subjects and subjected to T-lymphocyte subset analysis by fluorescence-activated cell sorting (FACS; Epics 753 with argon laser and triple fluorescence capacity; Coulter, Hialeah, FL) with the Ortho series of monoclonal antibodies, in which OKT8 identified the cytotoxic/suppressor subset of $CD8^+$ lymphocytes.

Plasma glucose concentration during the clamp was measured with a glucose oxidase system on a glucose analyzer (Beckman). Insulin was measured by the Herbert et al. modification (20) of the assay that was originally described by Yalow and Berson (21).

Student's *t* test was used to analyze the data. All data are expressed as means ± SE.

RESULTS

Euglycemic clamp. The goal of the euglycemic clamp was to achieve steady-state elevations in immunoreactive insulin in patients from which T lymphocytes were obtained while maintaining basal glucose, thus removing this variable as a potential receptor regulator. This goal was met as demonstrated in Table 1. Mean clamp glucose concentration from 0 to 240 min in normal and obese subjects was 5.11 ± 0.02 and 5.36 ± 0.01 mM, respectively, which was not statistically different from the fasting value and maintained with a coefficient of variation for the 4-h study of 4.3 ± 0.7 and 3.0 ± 0.3%, respectively. Whereas, plasma glucose was clamped at fasting concentrations, immunoreactive insulin was im-

portantly elevated in the normal and obese subjects to 602.4 ± 13.2 and 666.7 ± 19.2 pM, respectively (Table 1).

Insulin-receptor characteristics. Four hours of hyperinsulinemia created by the clamp technique substantially reduced the number of insulin receptors induced to appear on cultured T lymphocytes by alloantigen presented in the MLC in normal subjects, as previously described (13). Single-point insulin binding decreased in all normal subjects from 8.2 ± 1.2 to 2.5 ± 0.6 pg/10^6 lymphocytes ($P < 0.001$) by the 4th h of hyperinsulinemia, which was shown by Scatchard analysis in two subjects to be entirely a consequence of a reduction in the number of receptors appearing after lymphocyte activation. Insulin-receptor sites decreased from 6752 to 1665 sites/cell in these normal subjects in response to clamp-induced hyperinsulinemia, a decrease of nearly 75%, quite comparable to that previously reported (13). In contrast, there was no difference in single-point binding after 4 h of hyperinsulinemia in the obese cohort (3.12 ± 0.2 pg/10^6 lymphocytes at time 0 and 3.10 ± 1.2 pg/10^6 lymphocytes at 4 h). The time-0 preclamp number of sites ascertained by Scatchard analysis was 4502 (Fig. 1), a preperturbation value 33% of that measured in the two normal subjects, reflective of the reduced binding found in obesity reported in earlier studies by our laboratory (11,12). Interestingly, there was no further decrease in receptor sites after 4 h of acute hyperinsulinemia created on top of the chronic hyperinsulinemia that characterized this group of obese subjects.

MLC and LMC reaction. Lymphocytes removed from normal and obese subjects exposed to an in vivo hyperinsulinemic stress and placed in identical culture conditions exhibited no statistical alteration in the alloimmune response as adjudged by the MLC. In normal subjects, lymphocytes removed after 4 h of hyperinsulinemia exhibited [^{3}H]thymidine incorporation of 95 ± 9% of the incorporation effected by allostimulated T lymphocytes removed at time 0, before the institution of hyperinsulinemia (Fig. 2). In obese subjects, the 4-h incorporation level was trivially and not statistically different from basal (111 ± 23%; Fig. 2). Thus, despite measured reductions in insulin receptors on T lymphocytes removed from hyperinsulinemic normal subjects, alloantigen responsiveness was unchanged. These data support the view that, similar to the lack of effect of insulin concentrations on alloantigen responses of T lymphocytes in the MLC (8,18), such MLC responses are also independent of the number of lymphocyte insulin receptors on the cell membrane.

The stimulation index (SI), another construct used to evaluate quantitative alloimmune response in the MLC, was also analyzed in these studies. The SI is the ratio of the [^{3}H]thymidine incorporation measured after 6 days of MLC in the allogeneic test condition to the syngeneic control condition (SI = allogeneic cpm/syngeneic cpm; cpm, counts per minute) and permits analysis of the actual strength of

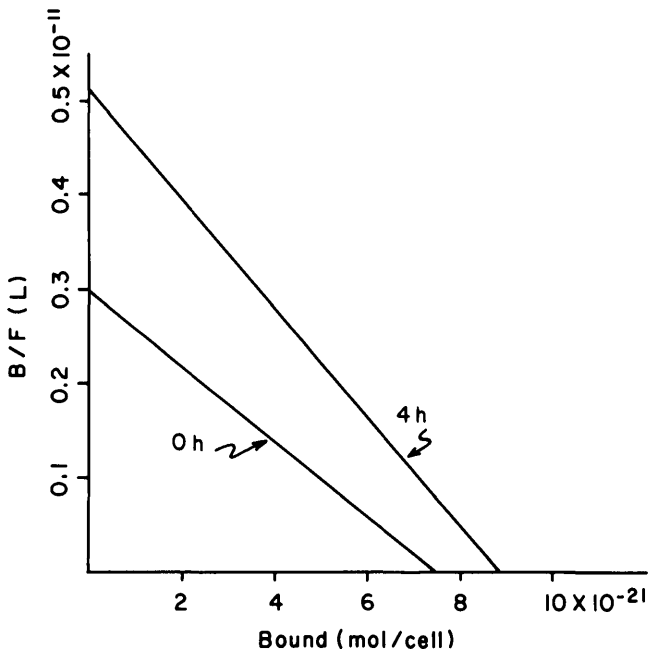

FIG. 1. Scatchard analysis of representative association binding isotherm to T lymphocytes removed at beginning and end of 4-h hyperinsulinemic-euglycemic clamp from obese subject. Least-squares analysis for binding data to time-0 lymphocytes predicts straight line with equation $y = -0.040 \times 10^{10}\ M^{-1} + 0.30 \times 10^{-11}$ L ($r = 0.93$), which gives K_d = 2.5 nM and 4502 sites/cell. Binding to 4-h cells gives $y = -0.059 \times 10^{10}\ M^{-1} + 0.051 \times 10^{-11}$ L ($r = 0.93$), which gives K_d = 1.7 nM and 5206 sites/cell.

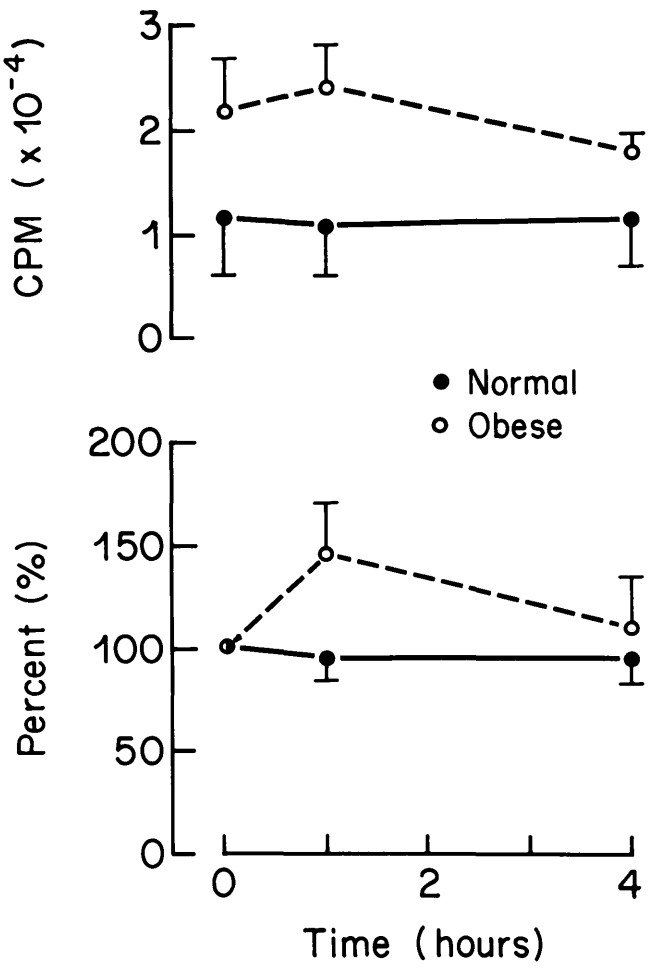

FIG. 2. Alloresponsiveness of T lymphocytes removed from hyperinsulinemic glucose-clamp conditions from normal individuals and chronically obese subjects. *Top*, uptake of [^{3}H]thymidine from cells; *bottom*, percent change from T lymphocytes removed from preclamp basal euinsulinemic conditions in subjects. cpm, Counts/min.

the alloimmune response basally and after removal of the T lymphocytes from subjects undergoing the glucose clamp. T lymphocytes from healthy subjects presented with alloantigen at time 0, before the institution of the clamp, exhibited a strongly positive response with an SI of 11 ± 5. Hyperinsulinemia induced by clamp, which was associated with a major reduction in activated T-lymphocyte insulin-receptor numbers, did not alter this basal alloresponsiveness: SI was 10 ± 4 at 1 h and 11 ± 4 at 4 h of clamped conditions (Fig. 3). Similarly, there was no impact of clamp-created hyperinsulinemia in the obese subjects over 4 h on alloantigen response adjudged by the SI (8 ± 3 at time 0, 9 ± 2 at 1 h, and 7 ± 2 at 4 h; $P > 0.05$; Fig. 3).

The LMC is an assay of cytotoxic T-lymphocyte response to targets bearing sensitizing antigens, which was chosen for study of acute insulin-derived receptor regulation on immune function because it is known to be regulated in part by insulin concentrations within the physiological range (1,4). Specific Cr release was 14 ± 4% in normal subjects at time 0 when they were euinsulinemic. Despite fixed and very low insulin concentrations in the in vitro tissue-culture environment in which the LMC was tested, sensitized cytotoxic T-lymphocyte killing was markedly reduced when the T lymphocytes were removed from subjects after 4 h of in vivo hyperinsulinemia and placed into the culture (reduced to 2 ± 2% sp Cr release, $P < 0.02$). In stark contrast was the absence of clamp-related changes in cytotoxic T-lymphocyte function observed in assays of cells removed from obese subjects (Fig. 4). Before the clamp, sensitized lymphocytes from obese subjects exhibited 10 ± 3% sp Cr release when harvested and tested 6 days later and placed in culture, which tended to be below the basal killing response of T lymphocytes obtained from normal subjects. Although we observed a small decrease in killing in lymphocytes removed from patients provided a hyperinsulinemic milieu at 4 h of clamp (7 ± 2% sp Cr release), this decrease was clearly not statistically significant.

T-lymphocyte subsets. To determine the impact of hyperinsulinemia on peripheral blood distribution of various T-lymphocyte subsets in general and the cytotoxic/suppressor pedigree ($CD8^+$) in particular, T lymphocytes were isolated

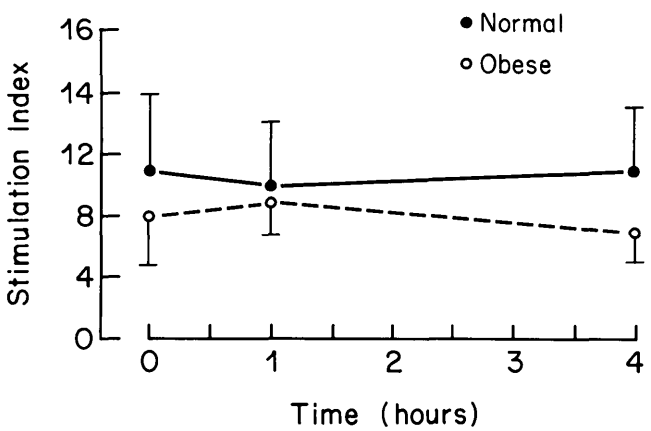

FIG. 3. Alloresponsiveness of T lymphocytes removed from normal and obese subjects in whom hyperinsulinemia was created in vivo by glucose clamp conditions depicted in terms of stimulation index, i.e., ratio of [^{3}H]thymidine uptake in allogeneic conditions to that from syngeneic conditions.

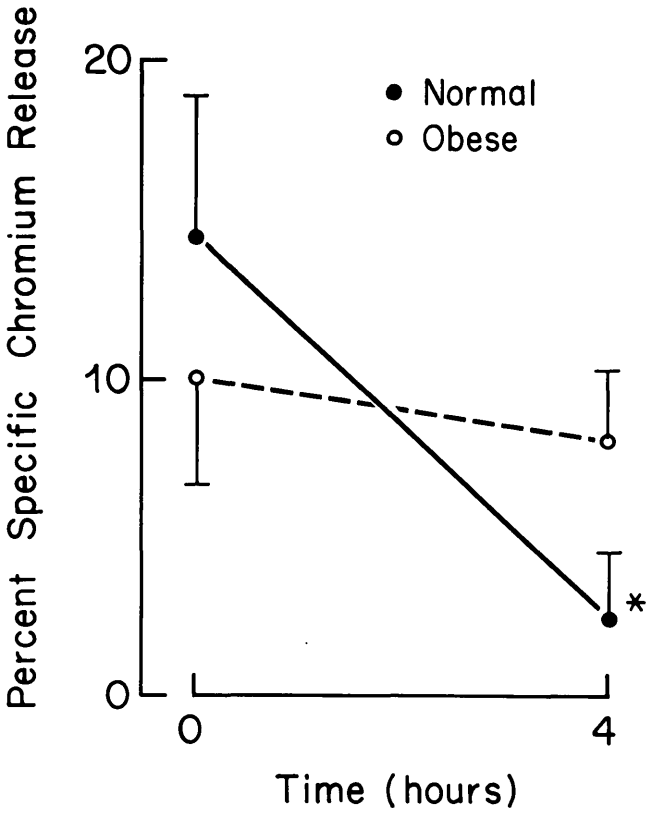

FIG. 4. Impact of in vivo hyperinsulinemia produced by glucose clamp on T-lymphocyte cytotoxicity reactions in tissue culture in cells from normal and obese subjects. *$P < 0.02$.

at time 0 and 4 h of the euglycemic clamp, and T-lymphocyte subsets were determined by FACS analysis of appropriate cell surface markers with each subject serving as his/her own control. Absolutely no differences were found in $CD8^+$ lymphocytes after 4 h of hyperinsulinemia in the normal subjects, who had a sharp reduction in insulin-supported LMC. At the initiation of the clamp study, there were 18 ± 5% $CD8^+$ lymphocytes in the peripheral blood and 17 ± 5% $CD8^+$ cells at the conclusion of the 4-h clamp.

DISCUSSION

We have been interested in understanding the hormonal regulation of immune function using insulin and its receptor as a model for study. We have shown that insulin is an important molecule that subserves an array of immunobiological activities. For example, insulin maintains the antigen-activated state of the lymphocyte when the insulin receptor is present on the cell membrane and thus is capable of engaging its ligand (8,10). Insulin has been shown by several laboratories to enhance the capacity of cytotoxic T lymphocytes to kill targets bearing the sensitizing antigen and to do so in a dose-dependent manner within the physiological range (1,4). Insulin can also function to enhance intracellular energy necessary to respond, in part, to the demands of activated lymphocytes through intercession of the binding event to its receptor on both T and B lymphocytes (9,19). This study tested the overall hypothesis that many of those immunobiological activities under the aegis of insulin regulation are modulated at the level of receptor display rather than at the level of alterations in the ligand concentration in

vivo. This hypothesis makes teleological sense, because insulin itself is under homeostatic control by mechanisms other than those relevant to immune function.

The regulation of the display of the insulin receptor on activated T lymphocytes can be characterized by three distinct mechanisms. *1*) Antigen or lectin can provide an on-off signal for de novo synthesis of the insulin receptor, the first event of which is translation of nuclear mRNA (2–4,7). *2*) The downregulation mechanism: the provision of the ligand to T lymphocytes already bearing their full complement of insulin receptors leads to diminution in the number of measurable surface receptors (22). *3*) The memory mechanism: studies from our laboratory revealed that circulating receptor-negative T lymphocytes perceive both chronic and acute alterations in ambient insulin and remember this information in tissue culture. This concept was studied further by this laboratory to elucidate the mechanism and the importance of the memory mechanism (12,13,15).

To test the hypothesis that insulin-receptor regulation has immunobiological consequence, we employed a strategy in which we used two groups of subjects, one in which we could acutely reduce receptor density by acutely raising plasma insulin by the euglycemic clamp (normal subjects) and another in which receptor density would be stable despite the perturbation in plasma insulin (obese subjects). We reasoned that only the normal subjects should express a change in that immunologic function related to insulin in this paradigm. To test the specificity of the immune responses, we chose to use assays of immune function known to be either responsive or unresponsive to modulation by plasma insulin. In the actual immune assays, the concentration of insulin was uniform and very low for all test conditions and all subjects, so that the only variable was the in vivo environment from which the test lymphocytes were removed. Variant insulin-receptor densities on these T lymphocytes were set during the clamp; the observation that the insulin-responsive LMC was diminished in normal volunteers as the density of insulin receptors that could be stimulated by antigen presentation fell to 25% of baseline supports the initial hypothesis. This finding was observed in each of the subjects and in group data. That there was no change in cytotoxic/suppressor T-lymphocyte numbers induced by the clamp underscores the primacy of insulin-receptor numbers in explicating the decrease in LMC induced by clamped conditions. That the obese subjects with a basal reduction in receptor number, commensurate with a reduction in initial cytotoxic T-lymphocyte function, did not exhibit a statistical fall in LMC in the face of no further reduction in receptor display is further evidence for the hypothesis. Last, that neither normal subjects with decreasing receptor numbers nor obese volunteers with stable receptor densities exhibited alterations in lymphocyte response to alloantigen in the MLC, a function thought not responsive to insulin modulation, supports the specificity of the finding.

For the basic strategy employed to test the validity of our hypothesis, we had to be sure that the two immune phenomena had variant responses to insulin in the culture environment as postulated. Presentation of alloantigen to lymphocytes in the MLC has been used by us for many years as an "insulin-independent" model of study. Strom and Bang (18), with an artificial tissue-culture medium that utilizes an artificial serum substitute and is therefore serum free and devoid of all growth factors unless added by the investigator, revealed that insulin had a small effect on the MLC. Although Helderman et al. (8) confirmed these results with growth factor–free medium enriched by a serum substitute, they were able to show that insulin had absolutely no role in altering alloresponsiveness in insulin-depleted but otherwise complete medium, the system employed in this study.

All investigators studying the insulin model agree that cytotoxic T-lymphocyte function is modulated by insulin, validating this assay as applicable to test our hypothesis. During the presentation of alloantigen, a subpopulation of T lymphocytes with cytotoxic capability and appropriate antigen receptors are sensitized (23). When presented with cells bearing the sensitizing antigen, a series of reactions are initiated that will culminate in the destruction of the target itself (24). Several intracellular signal pathways have been shown to modulate the strength of T-lymphocyte cytotoxicity. The nucleotide system was perhaps the first pathway described to function in this sense. Increased cAMP concentrations or adenylate cyclase activity by hormone or neurotransmitters decrease cytotoxicity. Increases in cGMP or decreases in cAMP have the opposite effect (25). Other hormones such as insulin also affect cytotoxic T-lymphocyte function through nucleotides or as yet unidentified signal-transduction pathways (26). Thus, the assays chosen for this study have validity for the test of the hypothesis that insulin-receptor regulation subserves an immunobiological modulatory role.

The findings in this study lend credence to the importance of our previous observations concerning fine regulation of the insulin receptor on the cell membrane of activated lymphocytes. In the exploration of hormonal immunoregulation with insulin as the model hormone of study, we have clearly shown that the hormone carries immunobiological significance through intercession of binding to a nonconstitutive membrane receptor. These new studies now link the two sets of observations. Regulation of the insulin receptor on activated lymphocytes is of immunobiological consequence and is the mechanism by which insulin-dependent immune functions may be modulated in systems in which the hormone is under feedback control by signals that do not involve the immune system.

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