1). The large number of shallow dives between 0 and 20 m are impossible to interpret without finer resolution in our recorders or some direct observations at sea, or both. Their short duration (< 1 minute) and their clustered occurrence suggest they could be for either shallow feeding or more general diving activity, such as during travel from one place to another.

The deeper dives between 20 and 140 m are probably associated with hunting and feeding. These dives lasted from 2 to 5 minutes, and they were usually clustered as a series of dives, often with striking consistency for depth. For example, seal 2 made 13 dives between 110 and 140 m in a space of 3.6 hours. Each dive lasted from 3.3 to 3.4 minutes, and the interval between dives ranged from 6 to 30 minutes ($\bar{X}$ = 17 minutes).

The deepest dive measured was 190 m, nearly twice that previously reported for this species (*7*). The duration of this dive (5.4 minutes) was one of the longest recorded. In previous studies in which adult female fur seals were forcibly submerged, their tolerance was only 5 minutes (*8*).

The diving profile (the plot of depth against time) of the 190-m dive showed that little time was spent at any depth. The average rate of depth change was 70 m/min. This rate is about the same as that of Weddell seals when they are routinely diving to depths of 200 to 400 m (*9*).

Considerable behavioral, ecological, and physiological information may be gleaned from the two variables, depth and time. Although we have investigated only the northern fur seal, this method of study could be used with many other species of marine mammals.

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1 April 1976

Insulin Binding to Cultured Human Fibroblasts Increases with Normal and Precocious Aging

Abstract. *Specific and nonspecific [^{125}I]insulin binding and concentration of unlabeled hormone producing 50 percent competition with 1.0 nanomolar [^{125}I]insulin for specific binding sites correlated positively with age of fibroblast donors. Cells from four children with precocious aging—three with progeria and one with Rothmund syndrome—resembled those from the chronologically old.*

Cultured human fibroblasts can be used to study genetic-regulatory aspects of insulin binding in normal and abnormal states without ethical restraints. With stable diploid human cells several generations removed from donor neurohumoral influences, the principal experimental variable is considered to be the genetic endowment of the cell strain (*1*).

We found positive correlation between age of the fibroblast donor and the amount of insulin, present at 1.0 n*M* concentration, bound to specific receptor sites. The most significant correlations were between donor age and (i) binding

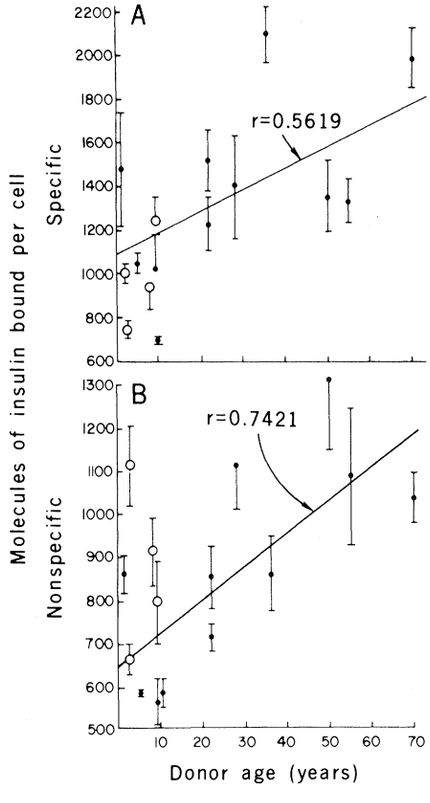

Fig. 1. (A) Correlation between specific binding of radioactive insulin to cultured fibroblasts and age of normal, nondiabetic cell donors ($P < .10$). Midpassage fibroblasts [generation 20 to 45, chosen by known lifespan of the strain (*12*)] were grown to confluence in 100-ml plastic dishes in Eagle's minimum essential medium supplemented with 15 percent fetal calf serum (*20*). Studies were performed a day after the monolayer reached confluence. All plates were examined for medium *p*H, confluence, and evidence of abnormality; subcultures were made from two plates to confirm vitality. Growth medium was aspirated from the plate and the cell layer washed twice with 6 ml of buffered saline at room temperature. Eagle's minimum essential medium (1.2 ml) with 1 percent bovine serum albumin buffered with 20 m*M* Hepes adjusted to *p*H 7 was added. ^{125}I-Labeled and native insulin were mixed with media before addition to the dish. After 45 minutes of incubation at room temperature the medium was removed for counting as the free fraction. The cells were scraped from the plate in 1 ml of buffered saline with a silicon-coated rubber scraper. The suspension of dislodged cells was withdrawn into a silicon-coated Pasteur pipette, placed in a plastic microcentrifuge tube, and centrifuged for 2 minutes at 10,000*g*; the supernatant was discarded and the cell pellet radioactivity was determined (*21*). [^{125}I]Insulin (50 to 100 μc/μg) was prepared from zinc-free pure bovine insulin (Connaught Laboratories) by the method of Hamlin and Arquilla (*22*). Material was kept lyophilized at −4°C and assayed for biological activity by the method of Gliemann (*23*), no loss of biological activity occurred for as long as 8 months under these conditions. Closed circles are normal strains; open circles are strains from precociously aged patients. Each point is the mean of three or more replicate experiments on different days; vertical bars, S.E.M. Specific binding is calculated from the difference between the radioactivity of cell pellets incubated with [^{125}I]insulin alone and those incubated in the presence of 100-fold native insulin. (B) Correlation between nonspecific insulin binding to cultured fibroblasts and age of normal donor ($P < .01$). Nonspecific binding was that fraction of [^{125}I]insulin added to the medium that remained bound to cells in the presence of a 100-fold excess of native peptide.

to nonspecific loci and (ii) $\frac{1}{2}B_{max}$, the concentration of native hormone necessary for 50 percent competition with [^{125}I]insulin for specific receptor sites (*2*); in these characteristics, fibroblasts from children with precocious aging resembled those from the chronologically old.

Cells were studied from 11 nondiabetic controls age 3 months to 70 years and from children 2 to 9 years of age with precocious tissue degeneration—three with progeria (*3*) and one with Rothmund syndrome (*4*). Maximal competition for specific binding in all strains was achieved with unlabeled peptide concentrations 10 to 50 times higher than that of labeled insulin; the counts remaining, termed nonspecific binding, comprised an average 39 percent of total binding. This nonspecific binding was 80 to 90 percent complete after 1 minute of incubation. Specific binding reached equilibrium between 30 and 45 minutes at 20°C. There was no decrease in binding over 3 hours. When medium was removed from dishes after 45 minutes of incubation and placed on fresh cells, specific binding in the second plate was 90 percent of that in the original dish, a result indicating little insulin degradation; there was no difference between strains in rebinding capability of this conditioned medium. Reversibility of the binding reaction was demonstrated by the removal of medium containing [^{125}I]insulin only (1.0 n*M*) from the monolayer at the end of incubation and reincubation with fresh medium without labeled insulin and with or without 100 n*M* unlabeled insulin. In five replicate experiments with three strains, this resulted in dilution of medium radioactivity by 90 percent. Without native hormone there was only 55 to 70 percent reduction in specific binding; with hormone the reduction was 70 to 85 percent. This reaction also reached equilibrium at 30 to 45 minutes. Glucagon, prostaglandin E_1, and A and B chains of insulin at concentrations up to 1.0 μM did not affect binding with 1.0 n*M* [^{125}I]insulin (*5*).

All normal cell strains demonstrated 1.2 to 3.6 percent specific binding of 1.0 n*M* [^{125}I]insulin (150 μU/ml) per 10^7 cells, or 650 to 2100 molecules of insulin per cell (Fig. 1A). This specific binding was independent of the amount of total protein (*6*) in the monolayer, protein content per cell, or cell density at confluence. There was positive correlation between binding and donor age ($P < .10$) (Fig. 1A).

Nonspecific binding in 1.0 n*M* [^{125}I]insulin varied from 0.94 to 2.45 percent per 10^7 cells, or 560 to 1300 molecules per cell (Fig. 1B); nonspecific binding correlated with donor age ($P < .01$), with protein content per cell ($P < .01$), and inversely with cell number in the confluent monolayer ($P < .001$). As with specific binding, there was no correlation with the amount of total protein in the dish.

Protein content per cell correlated with donor age ($P < .10$); the amount of total monolayer protein was independent of age. In cells from donors with precocious aging, protein content per cell differed from that in the four controls also under 10 years of age [precocious aging, 0.67 ± 0.24 pg (mean ± standard deviation); controls, 0.26 ± 0.07 pg; $P < .01$]. These cells also differed from those of child controls in number of molecules nonspecifically bound per cell in 1.0 n*M* [^{125}I]insulin (controls, 668 ± 129; patients, 876 ± 196; $P < .01$); cells from precociously aged subjects did not differ in specific binding (Fig. 1A) from those of young controls. They did differ in maximal number of cells attained at confluence (controls, $4.0 \times 10^6 \pm 1.2 \times 10^6$; patients, $2.6 \times 10^6 \pm 0.6 \times 10^6$; $P < .05$).

Dose-response curves were determined with [^{125}I]insulin alone at concentrations up to 1.0 n*M* and, above 1.0 n*M*, with varying amounts of unlabeled hormone (Fig. 2A). The dose-response curves were reproducible in cells of the same strain grown at different times and from different frozen stock. Individual characteristics of these curves included plateaus in the numbers of molecules bound with increased dose and, in the case of three normal and one progeria strain, two to four times as many insulin molecules bound per cell in 0.05 n*M* insulin (not shown) as in 0.1 n*M* insulin.

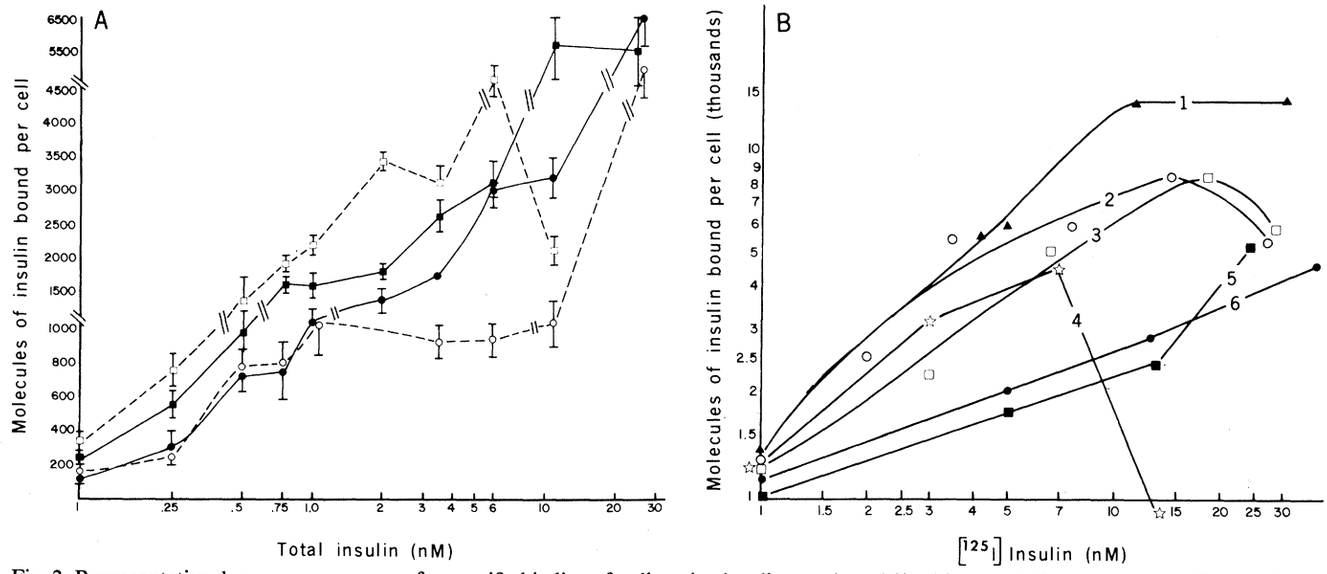

Fig. 2. Representative dose-response curves for specific binding of radioactive insulin to cultured fibroblasts. (A) Concentrations of 0.1 to 1.0 n*M* were labeled peptide only; those higher than 1.0 n*M* were achieved with appropriate amounts of unlabeled insulin together with 1.0 n*M* [^{125}I]insulin. Molecules of insulin bound per cell are calculated from the bound (*B*) and free (*F*) radioactivity by the formula: $(B/F)(M/6 \times 10^{23}C)$, where *M* is moles of insulin per milliliter and *C* is the number of cells. Cells are from two children age 3 months (closed circles) and 9 years (open circles) and two adults age 22 years (closed squares) and 36 years (open squares). Each point is the mean of three or more replicate experiments on different days and with different subcultures; vertical bars, S.E.M. (B) Saturation curves in six fibroblast strains exposed to [^{125}I]insulin; a log-log scale is used. For each point shown two confluent dishes were exposed to medium containing the indicated dose of [^{125}I]insulin, one with a 100-fold excess of native insulin added. Age of donor and $\frac{1}{2}B_{max}$ were as follows: No. 1, 50 years, 4.40 n*M*; No. 2, 10 years, 0.95 n*M*; No. 3, 22 years, 2.75 n*M*; No. 4, 9 years, 8.25 n*M*; No. 5, 5 years, 5.90 n*M*; and No. 6, 9 years, 1.21 n*M*. Strain No. 4 is from the patient with Rothmund syndrome, No. 5 from a child with progeria, and No. 6 from the same 9-year-old as in (A).

Because of this apparently complex and individual regulation of hormone-receptor interaction, linear analysis to estimate maximum binding capacity was felt to be inappropriate (*7*).

Saturation with labeled insulin alone proved possible in four of six strains in which it was attempted (Fig. 2B); 4,500 to 14,000 specific binding sites were estimated per cell. Two of these curves (Nos. 1 and 2) rise more steeply and higher than saturation curves obtained with increasing quantities of native hormone, while three (Nos. 3 to 5) coincide and one (No. 6) rises more steeply but no higher. Each point beyond 1.0 n*M* on the saturation curve with [^{125}I]insulin only is a single observation, in contrast to the thrice or more repeated replicate displacement studies; thus, the differences may not be important. Were there differing binding affinities for iodinated versus native hormone (*8*), the greatest discrepancies between saturation of labeled insulin alone and of labeled plus native insulin should be seen with strains requiring the highest concentrations of unlabeled hormone to produce 50 percent displacement. Data for five of the six strains in Fig. 2B fail to conform to this expectation.

The dose-response curves indicate that, in normal strains, saturation probably occurred at insulin concentrations of between 10 to 50 n*M* and half-saturation at those of 2.5 to 7.5 n*M*. There was no correlation between donor age and apparent maximum amount of insulin bound per cell in the displacement curves (in 10 or 25 n*M* total insulin). The $\frac{1}{2}B_{max}$ for each normal cell strain, ascertained from displacement curves, varied from 0.90 to 4.4 n*M* and correlated with donor age ($P < .01$) (Fig. 3). The $\frac{1}{2}B_{max}$ for the four patient strains (5.6 ± 2.0 n*M*, mean ± standard error of mean) differed markedly ($P < .005$) from that for the four control youngsters (1.00 ± 0.1 n*M*). The absence of age differences in apparent maximum binding and the presence of significant variation in $\frac{1}{2}B_{max}$ are similar to results for insulin binding in lymphocytes from obese and normal persons (*9*).

The replicative life-span of cultured fibroblasts is inversely related to the age of the donor (*10*). Precocious senility in the progeria syndrome is reflected in the decreased viability of fibroblasts cultured from affected persons (*11*); this in vitro senility was seen in this study as fewer cells at confluence and greater protein content per cell as compared to cells from other children. Progeria fibroblasts show profound defects in the HL-A surface membrane receptors for the immune system (*12*). Further evidence of widespread defects in protein synthesis (or degradation) is the increased heat lability of several enzymes from cultured progeria fibroblasts (*13*), similar to that seen with normal aging (*14*). The expression of the gene product insulin receptor may also be altered in precocious and normal aging.

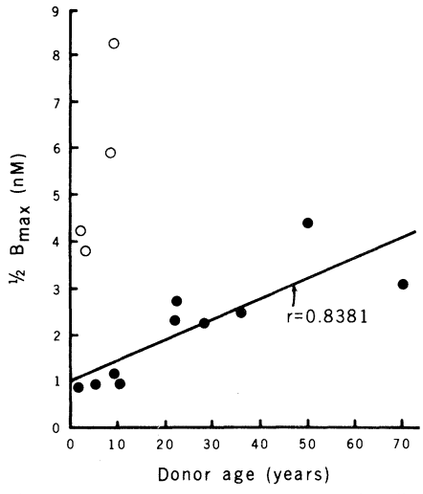

Fig. 3. $\frac{1}{2}B_{max}$ (concentration of native insulin required to displace 50 percent of [^{125}I]insulin specifically bound to cultured fibroblasts in medium containing 1 n*M* [^{125}I]insulin) as a function of age of normal donors ($P < .01$). Symbols are mean values for three or more experiments on different days and from different subcultures in replicate dishes with added native insulin at 1.0, 2.5, 5.0, 10, 25, 50, and 100 n*M*; closed circles, normal donors; open circles, precociously aged donors.

Progeria patients are resistant to the physiologic effects of insulin in vivo (*15*); this occurs with normal aging in humans (*16*) and fowl (*17*). Insulin receptor function in relation to biologic aging has not been previously described in humans. Decreased numbers of insulin-binding sites were found in skeletal muscle plasma membranes from older, fatter rats compared to young, lean ones; the dominant factor, however, appeared to be adiposity rather than aging (*2*).

We cannot say whether the significant increase in nonspecific binding with normal and precocious aging was causally related to monolayer density and protein content per cell, but it is likely that fewer cells with greater protein content would offer more surface and more nonspecific binding sites per cell. Specific binding per cell was independent of confluent density and cell protein content.

Concentration of unlabeled insulin causing 50 percent competition with 1.0 n*M* [^{125}I]insulin ($\frac{1}{2}B_{max}$) was the binding characteristic most significantly correlated with donor age and was most abnormal in the patients with precocious aging. That this was demonstrated in vitro, generations removed from the neurohumoral influences of the donor, indicates that these differences are genetically determined. Aging of the insulin binding system thus appears as increased affinity for insulin at levels that correspond to peak physiologic concentrations in vivo (*16*). This implies a requirement that more insulin molecules bind to achieve the same hormonal ends, consistent with greater insulin requirements in vivo with normal and precocious aging (*15, 16*).

Our data do not indicate whether the aging defect is accumulation of abnormal receptor protein, sluggish fluidity of the plasma membrane with altered interaction between occupied receptors, unoccupied receptors, and cyclic nucleotides (*7, 18*), or failure of activation of key intracellular events which could exercise feedback control of the hormone receptors (*7, 19*).

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24. Technical assistance was provided by E. K. Rosenbloom and E. Moerman. Supported by a faculty development award from the University of Florida, Medical Research Council of Canada, the Canadian Diabetic Association Foundation Fund, and the C. H. Best Foundation.
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Streptozotocin-Induced Pancreatic Insulitis: New Model of Diabetes Mellitus

Abstract. *Multiple small injections of streptozotocin in mice produce pancreatic insulitis, with progression to nearly complete beta cell destruction and diabetes mellitus. The timing and appearance of the inflammatory islet lesions suggest but do not prove that streptozotocin acts by initiating a cell-mediated immune reaction. Ultrastructural evidence of abundant type C viruses within beta cells of treated mice suggests that streptozotocin may activate murine leukemia virus in vivo in susceptible hosts.*

Streptozotocin (SZ) is a broad-spectrum antibiotic possessing antitumor (*1*), oncogenic (*2*), and diabetogenic (*3*) properties. The last action is mediated by pancreatic beta cell destruction and is widely used as a method for induction of diabetes in experimental animals and for clinical treatment of malignant beta cell tumors. For induction of experimental diabetes, SZ is conventionally administered as a single injection (*3*). After rapid clearance of SZ from the bloodstream [serum half-life is 15 minutes (*4*)], light microscopic evidence of beta cell necrosis is apparent within 24 hours (*5*). Beta cell necrosis, however, is detected by conventional ultrastructural examination after 2 to 4 hours (*5*), and intramembranous particle depletion of beta cell plasma membranes is observed within 45 minutes in freeze-fracture studies (*6*). Dissolution and phagocytosis of necrotic cells is rapid, with virtually no evidence of debris or inflammation visible after 3 days (*3, 5*). In a correspondingly rapid manner, blood glucose values peak 1 to 2 days after SZ administration and remain elevated if the appropriate quantity of the agent is given (*7*).

We present evidence here that SZ, given intravenously or intraperitoneally to laboratory mice in multiple subdiabetogenic doses, the usual method of clinical administration (*1, 4*), induces (i) pronounced pancreatic insulitis, with eventual destruction of insulin-secreting beta cells and diabetes mellitus, and (ii) enhanced replication of type C virus particles within pancreatic beta cells. The timing and appearance of the inflammatory islet lesions suggest but do not prove that SZ may initiate a cell-mediated immune reaction directed against the beta cells. The relevance, if any, of the increased number of type C virus particles to the inflammation and beta cell destruction is unknown.

Forty adult male mice (Charles River CD-1 strain), allowed free access to food and water, received five daily intravenous or intraperitoneal injections of SZ (40 mg per kilogram of body weight) (*8*) dissolved in a citrate buffer, *p*H 4.2, just before injection. A total of 43 uninjected mice and animals receiving equal volumes of citrate buffer were controls. Blood samples for glucose determination were collected in heparin-treated pipettes by orbital sinus puncture and assayed as described (*9*). Samples were obtained before each of the five daily injections and at frequent intervals afterward until animals were killed 6 days after the last injection. Four mice were also killed 12, 16, and 25 days after the completion of the SZ injections. The unpaired *t*-test (*10*) was used in statistical analyses. Pancreatic tissue obtained at death was fixed for light and electron microscopic study as described (*11*).

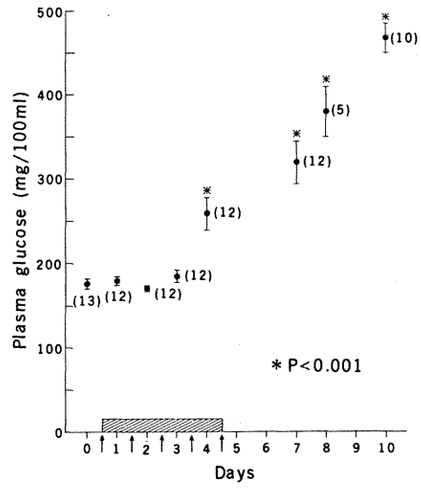

Fig. 1. Plasma glucose response in Charles River CD-1 mice after five intraperitoneal injections of SZ (40 mg/kg). The mean glucose value was significantly increased over the preinjection value (day 0) after the fourth dose of SZ and continued to increase progressively until termination of the experiment. Virtually identical results were obtained in 24 mice given intravenous injections of SZ (data not shown).

Plasma glucose values for SZ-injected mice were significantly elevated after the fourth injection (*12*) and increased substantially during the subsequent 6 days (Fig. 1) (*13*). Examination by light microscopy (Fig. 2) revealed large numbers of lymphocytes, moderate numbers of macrophages, and rare neutrophils surrounding and permeating the islets of Langerhans, with distortion of architecture and beta cell necrosis. Surviving beta cells were variably degranulated and the islets were generally smaller. Islet inflammation gradually diminished in animals killed 12, 16, and 25 days after the completion of injections [mean plasma glucose values of 396 mg/100 ml (N = 12), 525 mg/100 ml (N = 7), and 657 mg/100 ml (N = 7), respectively], and the remaining islets were small and composed almost exclusively of alpha and delta cells.

Ultrastructural studies of the islets in ten mice killed 6 days after the completion of injections revealed occasional necrotic beta cells and numerous infiltrating lymphocytes and macrophages. Unexpected, however, was the presence of large numbers of type C virus particles (*14*) within many intact, partially degranulated beta cells (Fig. 3B). Alpha and delta cells were normal. The pancreatic islets of uninjected mice and mice injected with citrate buffer appeared normal when examined by light and electron microscopy. Only an occasional virus particle was observed within the usually well-granulated beta cells (Fig. 3A). Quantitative morphometric studies of the number of virus particles per cell and the frequency of cells harboring viruses were not performed. Viruses were observed neither in the alpha and delta cells nor in the inflammatory cells.

In an effort to ascertain the timing of the appearance of the inflammatory cells in and around the pancreatic islets, animals were killed at daily intervals after having received one, two, or three intraperitoneal injections of SZ (40 mg/kg).