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DETECTION OF INSULIN AND INSULIN-LIKE GROWTH FACTORS I AND II IN SALIVA AND POTENTIAL SYNTHESIS IN THE SALIVARY GLANDS OF MICE

EFFECTS OF TYPE 1 DIABETES MELLITUS

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Abstract—The salivary glands of mammals synthesize and secrete a number of peptide growth factors that play important roles in cell/tissue homeostasis and embryonic development. Using a radioimmunoassay, insulin, insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) were detected in saliva from mice. Unlike epidermal growth factor (EGF), there was no sexual dimorphism in the concentrations of the insulin growth factor family. Immunohistochemical localization of IGF-I and IGF-II was confined to the duct cells of both the parotid and the submandibular glands. Reverse transcriptase-polymerase chain reaction amplification of total RNA from parotid and submandibular glands confirmed the presence of all three hormone/growth factor mRNAs in both glands. The levels of insulin and IGF-I were higher in saliva from an animal model for autoimmune type 1 diabetes, the non-obese diabetic (NOD) mouse, than in a second inbred strain, BALB/c. In contrast, the IGF-II levels were decreased relative to the BALB/c strain. With the onset of diabetes in NOD mice, insulin levels declined, while IGF-I and IGF-II levels showed trends toward lower levels of these growth factors when compared with non-diabetic animals. These changes were reflected in the concentrations from parotid and submandibular gland cell lysates.

Key words: growth factors; salivary glands; saliva; diabetes; RT-PCR; protein biosynthesis

Insulin and insulin-like growth factors are highly homologous growth hormones found in animal serum, which can generate similar biological responses in a wide variety of cell types [1–3]. Growth factor/hormone ligands initiate these responses by binding to their specific cell surface receptors. This interaction typically induces autophosphorylation of the receptor through intrinsic tyrosine kinase activity [1, 4, 5]. The increase in receptor-associated tyrosine kinase activity leads to the cascade of phosphorylation events necessary to generate the intracellular second messenger signal required for biological cellular responses [5–7].

The salivary glands of mammals appear to produce an array of biologically active peptides that are released into saliva upon stimulation of glandular exocytosis [8]. While the most widely recognized are the growth factors EGF¶ and NGF, saliva also has been shown to contain TGF_{α} and TGF_{β}, insulin and IGF-I and -II [9–16]. The synthesis of several of these growth factors has been confirmed to take place within the duct cells of the submandibular and parotid glands [11, 13, 16, 17]; others such as EGF and NGF are restricted in their synthesis to the submandibular gland in rodents [9, 10]. Furthermore, in mice, EGF additionally demonstrates androgen-dependent synthesis with male mice producing 100- to 500-fold higher levels of growth factor in the submandibular gland and saliva than do female mice [18].

Many of the growth factors/hormones have been implicated as mediators of a number of biological processes. Histochemical studies on the distribution of growth factor immunoreactivity have shown multiple sites of synthesis in fetal and adult tissues [19–22]. Synthesis in such varied organs as the lung, pancreas, kidney, liver and submandibular gland imply paracrine/autocrine as well as endocrine function for growth factors. EGF-like growth factors secreted in saliva have a systemic effect on wound-healing processes *in vivo* [23–25]. EGF is known to

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¶ Abbreviations: EGF, epidermal growth factor; NGF, nerve growth factor; IGF-I and IGF-II, insulin-like growth factor-I and -II; RT-PCR, reverse transcriptase-polymerase chain reaction; NOD, non-obese diabetic; PMSF, phenylmethanesulfonyl fluoride; IL, interleukin; TGF_{α} and TGF_{β}, transforming growth factor alpha and beta; RIA, radioimmunoassay; rh, recombinant human; mRNA, messenger ribonucleic acid; cDNA, copy deoxynucleic acid; and DM, diabetic.

affect the mucosal integrity of the gastrointestinal tract, rates of cutaneous and oral wound healing, and liver regeneration [23, 25–27]. The removal of the submandibular gland (sialoadenectomy) effectively causes a delay in tissue regeneration that can be corrected by supplementation of animal models with EGF.

There have been relatively few reports concerning the effects of pathological conditions on the saliva levels of growth factors/hormones produced by the salivary glands. Investigators using chemically induced insulin-dependent diabetes, genetic models for obesity-associated diabetes and autoimmune type 1 diabetes have observed decreased saliva concentrations of NGF and EGF in mice [28–30]. The levels of insulin in chemically induced diabetes have been reported to decline in both parotid and submandibular glands; however, the decrease was not as complete as the loss of pancreatic insulin due to β-cell cytotoxicity, suggesting that the salivary glands may represent an extrapancreatic source for this growth hormone. In the present report, we have evaluated the potential synthesis of insulin, IGF-I and IGF-II in the salivary glands and their concentration in saliva. Additionally, we have examined the effect of autoimmune disease type 1 insulin-dependent diabetes in an animal model, the NOD mouse, on the steady-state level of these three growth factors in saliva and salivary glands.

MATERIALS AND METHODS

Materials. Recombinant human IGF-I and IGF-II were purchased from Amersham Life Sciences (Arlington Heights, IL) as was ^{125}I-labeled growth factor and ^{125}I-labeled protein A. A guinea pig anti-porcine insulin, human IgG fraction and polyethylene glycol was obtained from the Sigma Chemical Co. (St. Louis, MO). Polyclonal antibody to Somatomedin-C (IGF-I) was obtained through the courtesy of Dr. A. F. Parlow, Harbor-UCLA Medical Center, as part of the NIDDK-supported National Hormone Distribution Program. Monoclonal antibody to rat IGF-II was purchased from UBI (Lake Placid, NY). BALB/c and NOD (Uf) mice were purchased from the Department of Pathology, University of Florida. Saliva collection and treatment of mice were performed in accordance with the University of Florida guidelines set forth by the Institutional Animal Care and Use Committee (Approval 2112). NOD mice were divided into those showing normal blood glucose levels (non-diabetic) and a second group with elevated blood glucose levels requiring daily insulin treatment (diabetic) [30]. Restriction endonucleases *Bst*YI, *Mbo*I and *Hae*II were purchased from Promega (Madison, WI). RIA of insulin concentrations in mouse saliva and tissue lysates was performed by Dr. Mark Atkinson, in the Department of Pathology Analysis Laboratory at the University of Florida. Poly(A)$^+$-RNA from the mouse pancreas was a gift from Dr. A. Peck, Department of Pathology, University of Florida. All other reagents for gel electrophoresis and histological evaluation were of ultrapure quality and purchased from commercial sources.

Removal and preparation of salivary glands. Mice were anesthetized by an injection of pentobarbital and killed by exsanguination of the heart; the parotid and submandibular glands were immediately placed into 10% PBS-buffered formalin for 6 hr at room temperature and subsequently stored in 70% ethanol at 4°. For RNA isolation, the fresh glands from BALB/c and NOD mice were homogenized in a solution of guanidine thiocyanate–phenol–chloroform according to the method by Chomczynski and Sacchi [31], as modified previously [32].

Paraformaldehyde-fixed samples for immunohistochemistry were serially dehydrated in ethanol and embedded in paraffin. The blocks were sectioned (5-μm thin sections) and incubated with a 1:250 dilution of antibody to insulin, IGF-I or IGF-II. After a 2-hr incubation in primary antibody, the slides were washed in PBS and incubated for 1 hr in a rabbit anti-guinea pig, goat anti-mouse or goat anti-rabbit biotinylated second antibody conjugate (Vector Laboratories, Burlingame, CA). The reaction was subsequently completed by incubation with a horseradish conjugate and the color reagent diaminobenzidine. The tissues were analyzed under light microscopy. As a control, tissue sections were reacted with preimmune serum from guinea pig and rabbit or a non-tissue reactive monoclonal antibody to IL-2.

Salivary gland tissues for RIA were removed, trimmed of fat and connective tissue, and homogenized in 10 mM Tris buffer, pH 7.5, containing 10 mM aprotinin, 4 μg/mL PMSF and 1.0 mM sodium orthovanadate [33]. The tissue lysate was clarified by extraction with 1.0 M acetic acid on ice for 30 min followed by centrifugation at 30,000 *g* for 30 min at 4° [11]. Protein assays were performed on all samples prior to RIA by the Bradford assay using BSA as a standard [34]. Prior to addition of samples for RIA, the proteins were separated from specific binding proteins present in saliva and gland lysates by heating to 65° for 5 min in the presence of 0.1% SDS.

Measurement of insulin, IGF-I and IGF-II concentrations in saliva. Whole saliva from female and male mice was collected by the method of Hu *et al.* [30]. Briefly, animals were given an intraperitoneal injection of pilocarpine (0.05 mg/100 g body wt) and isoproterenol (0.2 mg/100 g body wt) dissolved in saline. Animals were fasted for 18 hr before saliva collection. Diabetic NOD mice requiring insulin supplementation were removed from the hormone for 72 hr prior to saliva collection. Serum levels of insulin remaining in diabetic NOD mice were confirmed by RIA at the time of saliva analysis. Saliva collected by micropipette was placed into chilled microcentrifuge tubes.

Insulin levels were measured using a standard RIA performed in the Patient Analytical Laboratory of the Department of Pathology at the University of Florida. To obtain sufficient quantities of saliva, especially from xerostomic NOD mice [30], material was pooled from several days of collection of individual animals. In the case of BALB/c where normal saliva flow can be obtained, saliva was collected from a single period for individual animals to allow for the proper generation of displacement data in the RIA [11]. The detection limit for the RIA was between 10^{-11} and 10^{-10} M for each growth

factor or hormone. For both saliva and tissue, 1.0 mL of sample was diluted to 2.0 mL with sterile water, adjusted to 1.0 N acetic acid, incubated on ice for 30 min, and centrifuged to remove insoluble materials; then the supernatant was lyophilized [11]. Lyophilized saliva or salivary gland proteins were resuspended in 400 μL PBS for use in the RIA.

Saliva (200 μL) redissolved in PBS was incubated with 25 μL of a 1:50 dilution of the monospecific antibodies to IGF-I or IGF-II in PBS containing 0.1% BSA for 2 hr at 4°. ^{125}I-labeled IGF-I or IGF-II (60,000 cpm) in 25 μL was added and incubated for an additional 24 hr at 4°. Immune complexes were subsequently precipitated by the addition of 50 μL of human IgG (20 mg/mL) and 3.5 mL of 15% polyethylene glycol (mol. wt 6000) in PBS, followed by centrifugation at 7000 *g* for 20 min at 4° in a Sorvall RC5B (Norwalk, CT). Radioactivity in the pellet was quantified by a gamma counter. A standard curve for the growth factors and insulin was generated using dilutions of rh insulin, IGF-I and IGF-II. Displacement data were generated and linearized by logit transformation, and best-fit lines were determined by linear regression analysis [11]. The slopes of lines for growth factor/hormone standards and dilutions of saliva were compared for statistical differences using tests for slopes. Concentrations of IGF-I, IGF-II and insulin were calculated by averaging the amounts of growth factor/hormone interpolated from the linear regression curve of the standards and were expressed as nanomolarity for IGF-I and II or IU for insulin based upon the original volumes of saliva. All values for growth factor are the result of duplicated determinations from N = 8–11 animals. Student's *t*-test was used for statistical analysis of data. Concentrations are expressed as means ± SEM.

SDS–polyacrylamide gels and Western blot. As described previously for saliva analysis, 10 μL of whole saliva from BALB/c, non-diabetic and diabetic NOD mice was separated on a 15% SDS–polyacrylamide gel [30]. For the analysis of sexual dimorphism of growth factor/hormone expression, male and female BALB/c mice were analyzed. Saliva was placed into 0.5% SDS-containing sample buffer with 0.1% 2-mercaptoethanol, heated to 100° for 5 min, and applied to the well. After separation by size, proteins were transferred to polyvinyldifluoride nitrocellulose (Millipore, Bedford, MA) for 2 hr at 70 V [35]. The protein blots were blocked in a solution of Tris-buffered saline containing 3% gelatin and incubated at 23° in the same buffer containing a 1:500 dilution of the primary antibodies to insulin, IGF-I and IGF-II for 12 hr. The blots were washed three times and incubated for 2 hr with ^{125}I-labeled protein-A, washed, and exposed to Kodak XAR-5 X-ray film (Eastman Kodak, Rochester, NY). Purified bovine insulin rhIGF-I or rhIGF-II were included as a positive control for immunoblotting.

RT-PCR analysis of insulin, IGF-I and IGF-II mRNA. The ability of the salivary glands of mice to synthesize insulin, IGF-I and IGF-II was determined by specific RT-PCR amplification of total glandular RNA from the parotid and submandibular glands [11]. Briefly the RT-PCR reaction was performed as follows: 2 μg of total RNA was reverse-transcribed using a combination of oligo dT and random hexamer primers (Perkin-Elmer-Cetus, Emoryville, CA). The RT reaction included 10 min for annealing at 25° followed by extension at 42° for 1 hr, and then 95° for 5 min to inactivate the reverse transcriptase and RNasin. The primers for the reactions were as follows:

	Upstream	Downstream
Insulin	5′-AACTCCCAACCCTAAGTGACC-3′	5′-GTGGTGCACTCAGTTGCAGTA-3′
IGF-I	5′-TCACATCTCTTCTACCTGGC-3′	5′-CTCCTTAGATCACAGCTCCG-3′
IGF-II	5′-GCCTCCTCCTCTAATCTACCTC-3′	5′-GAGGACAGCGAGAGGCGGGTA-3′
β-Actin	5′-GTGGGCCGCTCTAGGCACCA-3′	5′-GAAGGTCTCAAACATGATCT-3′

Primers for IGF-I were provided by Dr. Maria Grant, Department of Endocrinology and Metabolism, University of Florida. Primers for insulin and IGF-II were generated from Genbank sequences and synthesized by the Interdisciplinary Center for Biotechnology Research Oligonucleotide Synthesis Core, University of Florida. Insulin cDNA was amplified, beginning with the addition of *Taq* polymerase with a 94° initial melt for 4 min followed by 50 cycles at 94° for 1 min, 55° for 2 min, and 72° for 3 min. IGF-I cDNA amplification was performed with a 94° hot start for 2 min, followed by 94° for 0.5 min, 60° for 1 min and 72° for 1.5 min for 35 cycles. IGF-II cDNA amplification was achieved by a 94° hot start for 4 min followed by 94° for 1 min, 59° for 2 min and 72° for 3 min for 40 cycles. The amplified products were visualized on an ethidium bromide-stained 1.5% agarose gel. The insulin, IGF-I and IGF-II primers should yield products of 521, 366 and 537 bp, respectively. Construction of primer and restriction endonuclease digestion sites was obtained through an analysis of a Genbank DNA sequence database. Confirmation of the synthesis of the correct amplicon was provided with restriction endonuclease digestion of the excised band corresponding to the proper PCR product size listed above. For RT-PCR quantitation of mRNA steady-state levels in BALB/c and NOD mice, the above protocol was employed with the following modification: in addition to the primers for IGF-I, PCR reaction mixtures contained primer pairs for β-actin, which served as an internal control. Following a random primer cDNA synthesis and a 4-min hot start at 94°, IGF-I and actin were co-amplified for 40 cycles at 94° for 1 min, 59.5° for 2 min, and 72° for 3 min [36, 37].

RESULTS

Whole saliva from BALB/c and NOD mice were assayed for immunoreactive insulin, IGF-I and IGF-II protein using specific RIA. Dilutions of mouse saliva contained material that competed for binding of ^{125}I-labeled growth factor/hormone with antibodies specific for these proteins. Logit

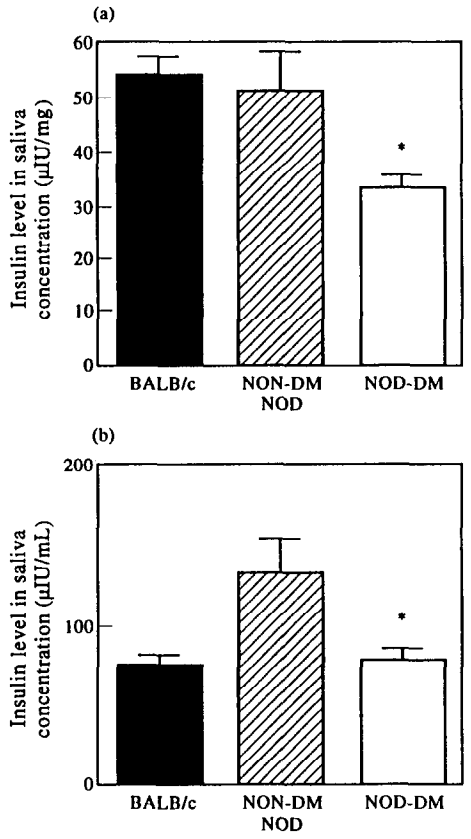


Fig. 1. Histogram of insulin concentration in BALB/c mouse saliva. Insulin levels are expressed μIU/mg protein (a) or μIU/mL saliva (b). Significant differences are indicated by an asterisk ($P < 0.02$) for NOD-DM mouse concentrations of insulin relative to non-DM NOD. Solid bars represent BALB/c mice, hatched bars represent non-diabetic NOD mice, and open bars represent diabetic NOD mice. Insulin concentration is expressed as means ± SEM; N = 12 for BALB/c, 12 for non-diabetic NOD, and 9 for diabetic NOD mice.

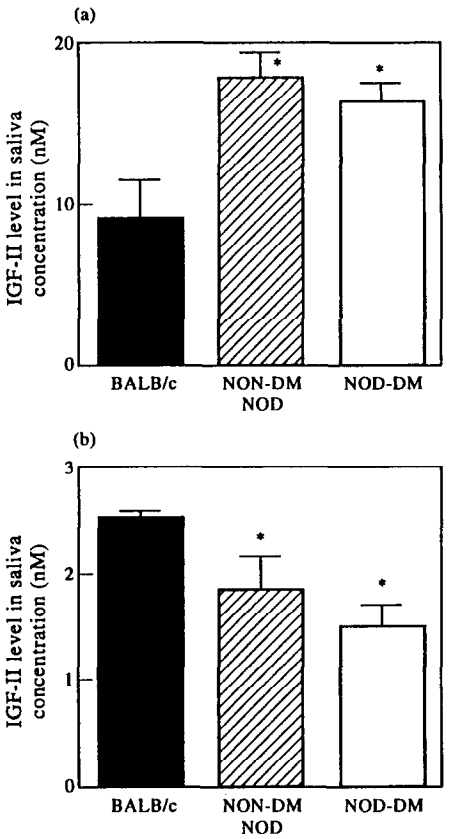


Fig. 2. Histogram of IGF-I (a) and IGF-II (b) concentration (molarity) in murine whole saliva. Significant differences are indicated by an asterisk ($P < 0.05$) for NOD mouse growth factor concentrations relative to BALB/c control. The bar code is defined in the legend of Fig. 1. Growth factor levels are expressed as means ± SEM; N = 6 for BALB/c, non-diabetic and diabetic NOD mice.

transformation of the displacement data generated lines with slopes that were not significantly different from the line generated by pure preparations of these proteins, indicating the presence of immunoreactive insulin, IGF-I and IGF-II protein in saliva (data not shown). The calculated level of insulin per unit volume saliva (Fig. 1) was similar to that reported in human saliva [38]. An analysis of saliva concentrations for insulin, based upon unit protein content, in NOD mice showed similar levels of hormone between non-diabetic and BALB/c animals that declined by 35% with the onset of diabetes (Fig. 1a; $P < 0.02$). When evaluated relative to unit volume (Fig. 1b), insulin levels in saliva were highest in non-diabetic animals (1.9-fold over BALB/c levels; $P < 0.01$) but declined with the onset of diabetes in NOD mice ($P < 0.05$). While serum levels of insulin in BALB/c and non-diabetic NOD mice were similar (37 and 42 μIU/mL, respectively), diabetic mice removed from insulin supplementation had serum insulin levels of approximately 25-fold less (1.63 μIU/mL). The concentration of IGF-I (Fig. 2a) in saliva was 2-fold higher in non-diabetic NOD mice than that present in BALB/c ($P < 0.01$). However, with diabetes onset in the NOD strain, there was a non-significant decline of IGF-I values in the mice. A third pattern of expression was observed in the RIA evaluation of IGF-II in mouse saliva. The level of this growth factor was lower in both the non-diabetic and diabetic state of the NOD strain when compared with BALB/c levels (Fig. 2b; $P < 0.05$). Again, there was a trend toward a decrease in concentration of IGF-II with diabetes onset, but it was not significant ($P > 0.05$).

The presence of immunoreactive growth factor/hormone was confirmed by Western blot analysis using an equal volume of saliva (Fig. 3). Using saliva from BALB/c mice, Fig. 3 shows the level of insulin, IGF-I and IGF-II in male and female saliva, along with the pure hormone or growth factor as a positive control. The level of members of the insulin-like growth factor family was similar for both male and female mice, indicating that there was no sexual dimorphism of expression. The immunoreactive insulin, IGF-I and IGF-II identified in saliva also

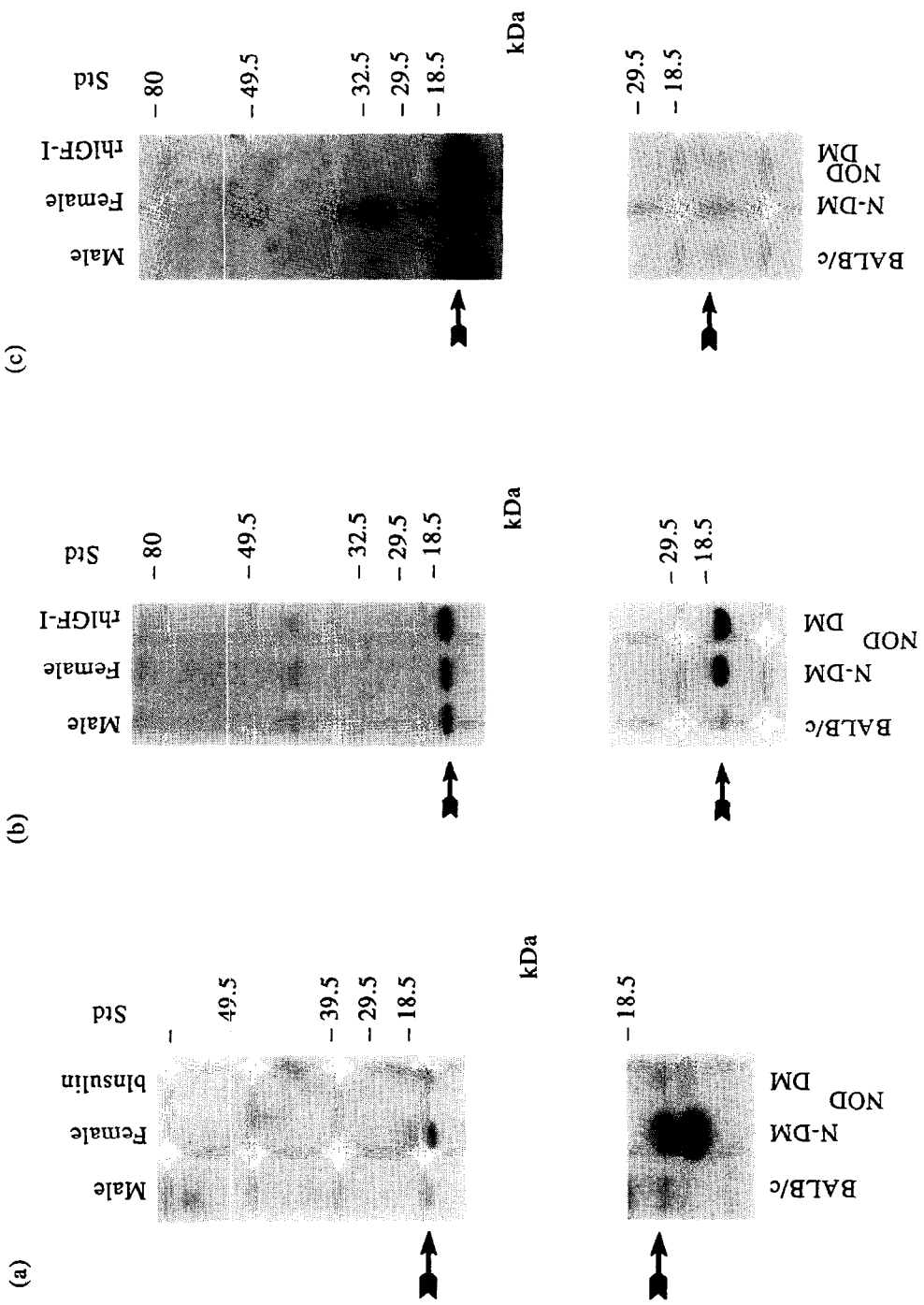


Fig. 3. Western blot analysis of insulin (a), IGF-I (b) and IGF-II (c) in mouse saliva. Ten microliters of whole saliva was separated on a 15% SDS-polyacrylamide gel, followed by transfer to polyvinyldifluoride membranes. The upper portion of each panel shows the results of male vs female saliva along with the identification of a positive control for each hormone/growth factor. Control proteins are bInsulin, bovine insulin; rhIGF-I and rhIGF-II, recombinant human IGF-I and -II. The bottom portion of each panel represents the analysis by Western blot of saliva used for RIA in Figs. 1 and 2. N-DM, non-diabetic NOD mice; DM, diabetic NOD mice. Each figure is representative of a profile of six individual salivas. Prestained molecular weight standards are: BSA, 80,000 Da; ovalbumin, 49,500 Da; carbonic anhydrase 33,500 Da; soybean trypsin inhibitor, 29,500 Da; and lysozyme, 18,500 Da. Arrows indicate the mobility of the monomeric form of the hormone/growth factor under denaturing conditions.

co-migrated with purified protein, indicating the secretion of the mature form of hormone/growth factor. In all, four male and four female mice were analyzed by Western blot with the same pattern of equal quantity of immunoreactive protein identified as represented in the example in Fig. 3. The changes in growth factor/hormone levels detected by RIA for BALB/c and NOD mice were confirmed in the western blot analysis of saliva (Fig. 3). For both IGF-II and insulin, there appeared to be the detection of a second protein species that was smaller than the mature form of the growth factor and most likely represented partial degradation of these proteins in saliva. Immunodetection of both bands was lost with the inclusion of the purified growth factor when preincubated with primary antibody, indicating that there was no cross-reactivity in the insulin-like growth factor family antibodies used in this study. When non-denatured material was separated on SDS–polyacrylamide gels, there was no indication of co-migration of growth factor with high molecular weight binding proteins (data not shown).

Since NGF, EGF, TGF_{α} and insulin have been shown to be present in the duct cells of the submandibular gland or the submandibular and parotid glands [10, 11, 17] of rats and mice, immunohistochemistry was performed to identify the sites of salivary gland biosynthesis of IGF-I and IGF-II. As shown in Fig. 4, immunolocalization of

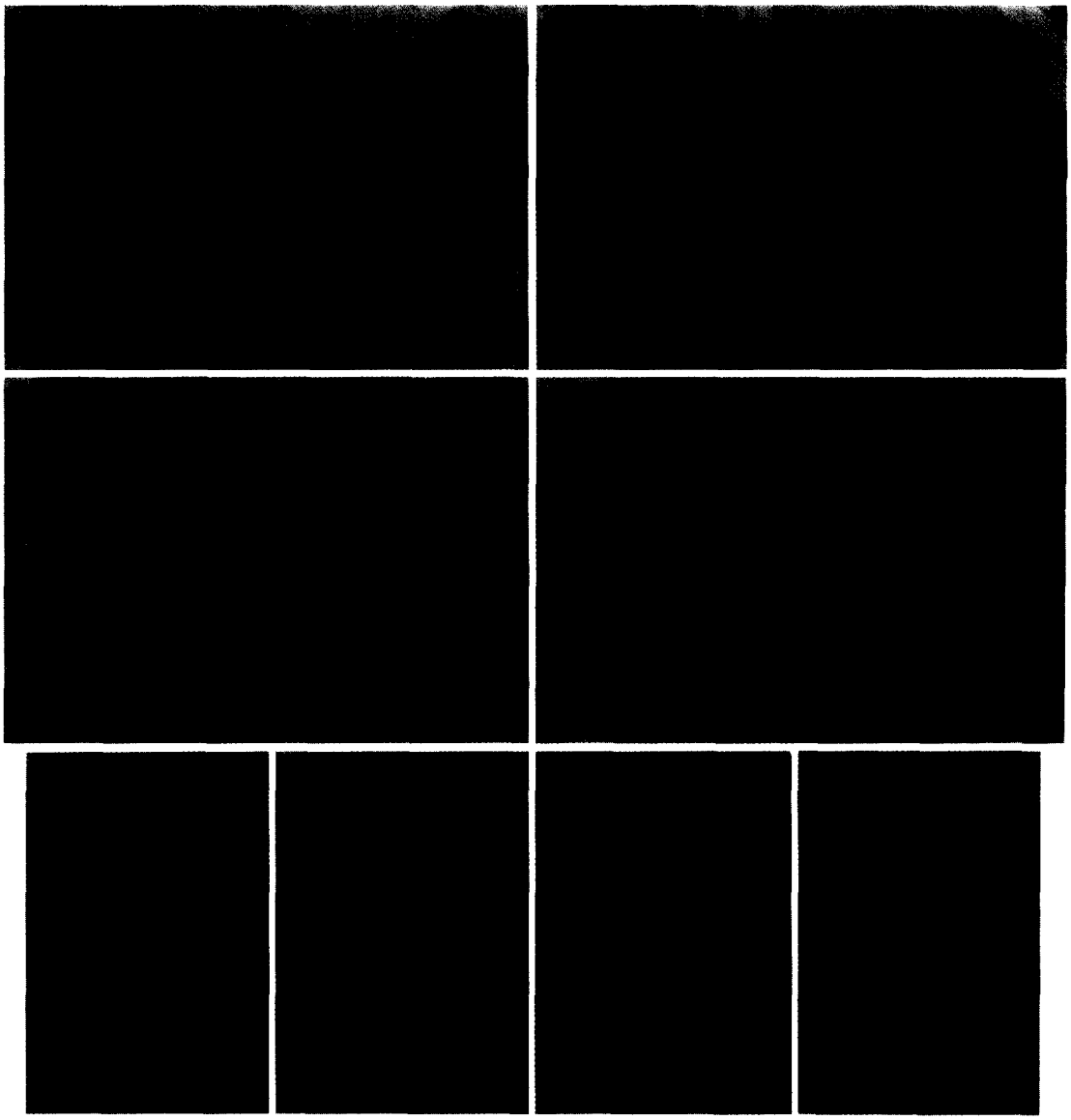


Fig. 4. Immunohistochemical localization of IGF-I and IGF-II in murine salivary glands. Polyclonal antiserum to IGF-I or monoclonal antiserum to IGF-II was diluted 1:250 and incubated with 5-μm thin sections of BALB/c parotid (PAR) or submandibular (SMX) glands, as indicated in Materials and Methods. The parotid and submandibular glands were also reacted with rabbit preimmune serum (P.I.) or a non-reactive monoclonal antibody to IL-=2. Magnification: 300×.

IGF-I and IGF-II in the mouse was associated primarily with the striated duct cells and granular tubule cells of the parotid and submandibular glands, respectively. The commercially available antibody preparation to insulin did not recognize any protein upon utilization in immunohistochemical analysis of salivary tissue. Thus, we were unable to confirm its presence in the gland although previous immunohistochemical localization and biosynthetic radiolabeling have suggested this hormone to be synthesized by the granular tubule cells of both the parotid and submandibular glands [13, 14].

To assess the ability of the salivary glands of mice to synthesize insulin, IGF-I or IGF-II protein, RNA was isolated for RT-PCR amplification. Using specific primers to mouse sequences for these growth factors/hormones, an expected product was observed to migrate at 521, 366 and 537 bp for insulin, IGF-I and IGF-II, respectively, in agarose gels (Fig. 5). In the case of insulin, RT-PCR of poly(A)$^+$ containing RNA from the pancreas produced an amplification product that co-migrated with the amplification products of the parotid and submandibular glands. The parotid gland also appeared to produce more mRNA for insulin than the submandibular gland, consistent with the observations of Patel *et al.* [17] based upon RIA. The authenticity of insulin mRNA in the salivary glands was confirmed by restriction endonuclease digestion with *Bst*YI, which yielded expected bands at 406 and 115 bp. Similar levels of IGF-I and IGF-II mRNA were detected for the parotid and submandibular glands following RT-PCR. In the case of IGF-II, multiple products were detected (Fig. 5). Confirmation of authentic IGF-I and IGF-II was obtained by restriction enzyme digestion with *Mbo*I and *Hae*II, respectively. Both enzymes gave predicted band sizes following the extraction and digestion of the amplicon of the expected size (see Fig. 5).

The changes in saliva levels for IGF-I and IGF-II were further analyzed by RIA of total gland lysates at 21 weeks of age. The histogram profiles revealed a similar pattern of either increased or decreased levels of growth factors in the cell lysates prepared from the parotid or submandibular glands (Fig. 6). Again, the level of IGF-I was higher in concentration in the non-diabetic NOD mice than in BALB/c and remained elevated with the onset of diabetes, whereas the IGF-II levels were lower in the NOD mice relative to the BALB/c strain. Since we were able to produce a single amplification product with RT-PCR using the IGF-I primers, we examined the mRNA for this growth factor in total RNA prepared from the parotid and submandibular glands of BALB/c and NOD mice. To quantitatively evaluate changes in the steady-state mRNA levels, PCR primers were also included for the co-amplification of β-actin mRNA [36, 37]. As shown in Fig. 7, the changes in protein levels in the gland lysates were not reflected in changes in steady-state mRNA levels for IGF-I in the NOD mouse salivary gland RNAs. The steady-state level of IGF-I mRNA was lower in proportion to β-actin in the glands by 60 and 73% in the diabetic (for the parotid and submandibular glands, respectively) NOD mice, when compared

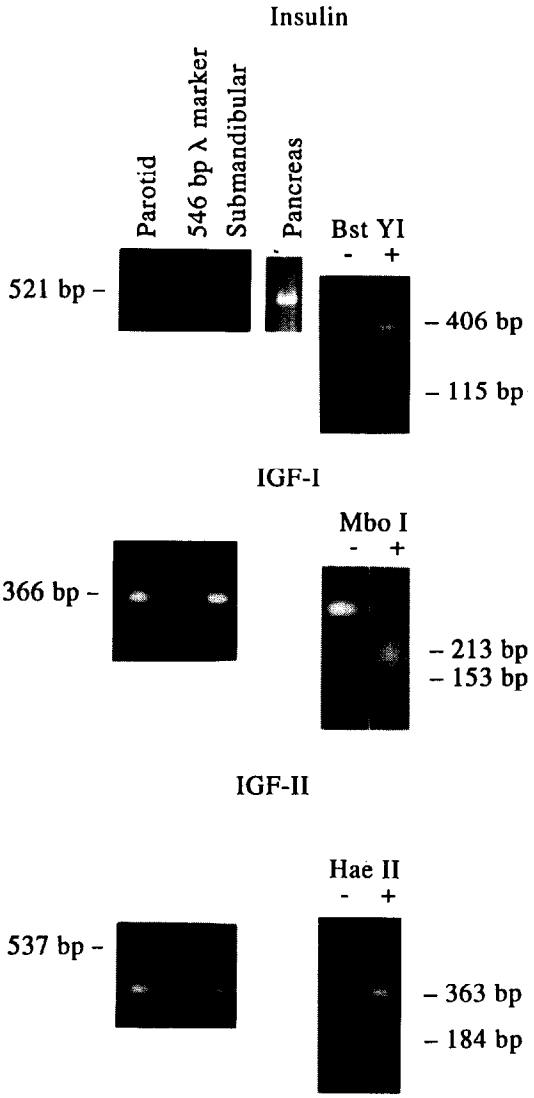


Fig. 5. RT-PCR amplification of insulin IGF-I and IGF-II mRNA. Total RNA was isolated from parotid gland, submandibular gland or pancreas. The ethidium bromide stained products of the RT-PCR reaction are shown to the left with the 564 bp band of Lambda *Hind*III cut DNA marker indicated in the middle lane between parotid and submandibular gland products. The right-hand side of the figure shows the expected restriction endonuclease digestion products for insulin, IGF-I and IGF-II by *Bst*YI, *Mbo*I and *Hae*II, respectively, following their excision from the gel and subsequent reseparation on a 2% agarose gel stained with ethidium bromide.

with non-diabetic animals ($P < 0.01$). On the other hand, IGF-I levels were almost 5-fold higher in the non-diabetic submandibular gland than in BALB/c mice, but the parotid gland of non-diabetic NOD showed a lower steady-state level than that of BALB/c mice (27% less; Fig. 7b).

DISCUSSION

The present investigation was undertaken to elucidate the synthesis of insulin, IGF-I and IGF-II

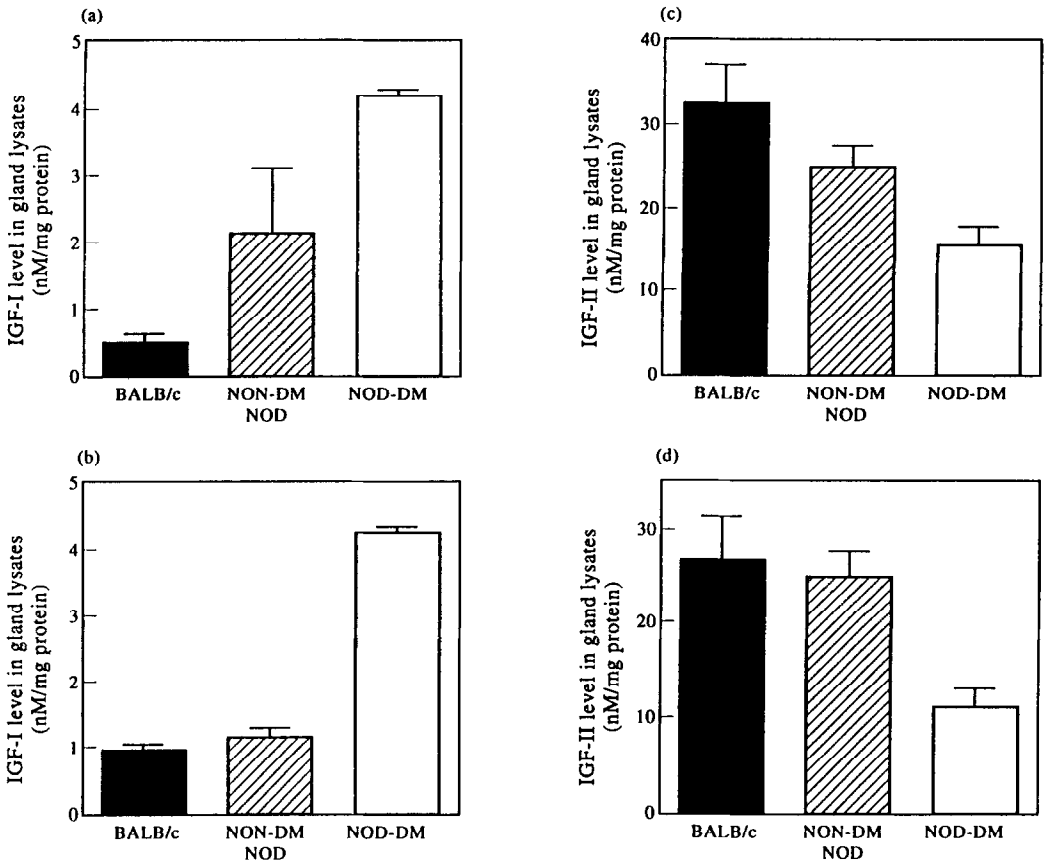
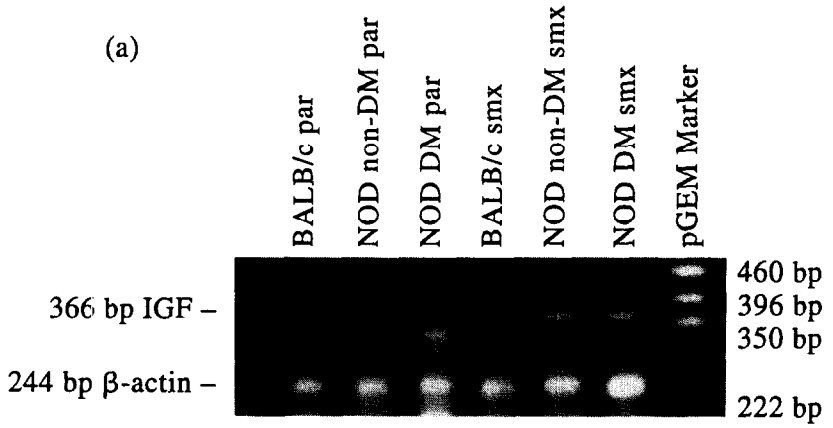


Fig. 6. Histogram of glandular levels of IGF-I and IGF-II in BALB/c and NOD parotid gland (a and c) or submandibular gland (b and d), respectively. All values are presented as means ± SEM. Solid bars represent BALB/c mice; hatched bars represent non-diabetic NOD mice; open bars represent diabetic NOD mice. Each experiment was performed in duplicate for N = 6 animals for BALB/c, non-diabetic and diabetic NOD mice.

by the salivary glands of mice. Insulin has been shown by immunohistochemical localization and RIA to be present in the duct cells of the parotid and submandibular glands from rats, mice and humans [13, 14, 17, 39]. Synthesis of insulin was confirmed through the detection of radiolabeled product from rat and human tissue incubated *in vitro* with [^{3}H]leucine [14]. The hormone has also been detected in human saliva and, therefore, it could be concluded that insulin is actively synthesized and secreted by the salivary glands. The results presented here confirm and expand these observations to the mouse through the detection of mRNA for insulin in salivary gland extracts and by the determination of immunologically reactive protein in saliva. Unlike EGF synthesis by the mouse submandibular gland, there was no evidence of androgen-dependent regulation of synthesis as determined by western blot analysis of saliva. This was also true for IGF-I and IGF-II. Interestingly, in an autoimmune model for type 1 diabetes, insulin levels in saliva decrease with diabetes onset when compared with non-diabetic NOD mice. The decline in hormone in saliva with diabetes onset was not nearly as dramatic as observed for the pancreas and suggests that the salivary glands may act as an extrapancreatic source of insulin. Since the level of insulin produced by the salivary glands is 10- to 20-fold lower than that produced by the pancreas [17], as well as needing to pass through the gut for absorption, it may not be possible for salivary insulin to have a systemic effect on regulating blood glucose levels. Thus, salivary insulin may have an autocrine effect on salivary tissue homeostasis.

While the biosynthesis of insulin and the presence of immunoreactive material in the ductal cells of both the parotid and submandibular glands have been shown previously [13, 14], controversy remains as to the origin of insulin in saliva. It has been suggested that there exists a linear relationship between serum and saliva levels of insulin [40, 41], which may be responsible for the observation of authentic hormone in saliva. The observation of mRNA for insulin along with the RIA results of saliva insulin levels again suggests that the presence of this hormone in saliva is most likely due to secretion of locally synthesized protein from the salivary glands. Removal of diabetic mice from insulin injection for 78 hr caused a near 25-fold decline in serum insulin levels, whereas saliva levels

(a)



(b)

	IGF-I/βActin[a] Densitometer ratio	
	PAR	SMX
BALB/c	0.319	0.039
non-DM NOD	0.233	0.191
DM NOD	0.142	0.045

Fig. 7. RT-PCR amplification of IGF-I in parotid and submandibular glands from BALB/c, non-diabetic NOD and diabetic NOD mice (a). The amplification of IGF-I produced a single band of 366 bp while co-amplification of the actin oligonucleotide primers produced a predicted product of 244 bp. The steady-state levels of IGF-I relative to β-actin were quantitated by densitometer analysis of three different RT-PCR reactions (b). The values represent the means for these analyses. Values are expressed in arbitrary area units. Key: (a) PAR, par = parotid gland; SMX, smx = submandibular gland.

of the hormone were reduced by only 0.5-fold from the non-diabetic NOD mice. Animals still receiving daily hormone injections showed the same levels of insulin in saliva as the mice not receiving insulin injections following an 18-hr fasting period (data not shown). The detection of mRNA for insulin in this study coupled with observations by others for immunohistochemical localization to the duct cells of insulin and the recovery of radiolabeled hormone following incubation of salivary cells with [^{3}H]leucine [13, 14] support the conclusion that insulin present in saliva is derived from salivary gland synthesis.

IGF-I and IGF-II were also detected in the duct cells of both salivary glands, as was the mRNA for these growth factors. While IGF-I can bind to the insulin-receptor and mediate many of the same cellular responses as insulin, IGF-II binds to a distinct receptor on various cell types. These two growth factors were found to show different patterns of concentration in saliva of NOD mice as compared with BALB/c mice, which most likely are the result of strain specific differences. However, while IGF-I levels were higher in non-diabetic NOD mice, IGF-II levels were lower than BALB/c with a trend toward a further decline with diabetes onset for both growth factors ($P < 0.05$). This pattern for IGF-I and IGF-II is similar to that reported for EGF in the NOD strain [11] as well as the chemically induced and obesity models of diabetes in mice [28, 29]. Steady-state IGF-I mRNA levels in the salivary glands of diabetic NOD mice were also reduced relative to the non-diabetic NOD animals. The distribution of IGF-I mRNA in BALB/c mice showed the parotid gland to be the predominant source of this growth factor while in NOD strain mice IGF-I mRNA appeared to be more equally synthesized by both the parotid and submandibular glands. It is also possible that using β-actin as a control RNA is inappropriate in the autoimmune disease state with the elaboration of lymphokines and cytokines by infiltrating lymphocytes, which may be affecting acinar or ductal cell function. Since IGF-I can bind to the insulin receptor, it is possible that this growth factor may compensate for the decline in salivary insulin levels in maintaining tissue homeostasis. In the mouse, the greater quantity of IGF-I over IGF-II in saliva is similar to that reported for human saliva [15].

It has now been shown by several different techniques that the salivary glands and saliva serve

as a reservoir for a large number of growth factors and hormones [8–12]. The biological significance of the presence of these products is unclear. However, animal studies involving sialoadenectomy have revealed that, at least for EGF, saliva loss for this growth factor produces a profound effect on systemic tissue homeostasis [42–44] and rates of wound healing and tissue regeneration [24–27]. In the case of insulin, it has been shown that this hormone can modulate salivary gland secretory processes [45, 46] as well as specific protein synthesis [47–49]. Thus, insulin and/or IGF-I may act in an autocrine fashion to maintain aspects of salivary gland function with the loss of pancreatic sources of the hormone [30]. Any evaluation of the potential for a paracrine role in maintaining oral health must await the analysis of the receptor distribution for these factors on oral tissues.

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