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Immunology

Elevated levels of cysteine protease activity in saliva and salivary glands of the nonobese diabetic (NOD) mouse model for Sjögren syndrome

(autoimmunity/apoptosis/protein processing/parotid secretory protein)

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ABSTRACT **Nonobese diabetic (NOD) mice develop an anti-exocrine gland pathology similar to human Sjögren syndrome. Recently, we demonstrated that NOD-*scid* mice develop severe loss of submandibular acinar cells with concomitant appearance of abnormal isoforms of salivary proteins suggesting *de novo* enzymatic cleavage. Because these changes may indicate activation of apoptotic proteases, we examined saliva and salivary tissue for cysteine protease activity. Cysteine protease activities were elevated in saliva and gland lysates from 20-week-old NOD and NOD-*scid* mice as compared with age- and sex-matched BALB/c or 8-week-old NOD mice. This activity appeared in the submandibular glands, but not in the parotid glands. Western blot analyses using antibodies directed against specific apoptotic proteases (interleukin 1β converting enzyme, Nedd-2, and Apopain/CPP 32) confirmed these findings. Submandibular glands from NOD-*scid* mice exhibited the greatest increase in proteolytic activity, indicating that infiltrating leukocytes are not responsible for these changes. Western blot analyses also failed to reveal changes in the levels of cystatins (saliva proteins that inhibit protease activity). Thus, increased cysteine protease activity appears to be directly related to submandibular acinar cell loss in NOD-*scid* mice involving the apoptotic pathway. Additional protease activity in saliva and gland lysates of older NOD and NOD-*scid* mice, apparently mutually distinct from cysteine proteases, generated an enzymatically cleaved parotid secretory protein. We suggest, therefore, that proteolytic enzyme activity contributes to loss of exocrine gland tolerance by generating abnormally processed protein constituents.**

The nonobese diabetic (NOD) mouse, a model for type 1 insulin-dependent diabetes, develops an autoimmune-dependent destruction of its pancreatic β cells leading to loss of blood glucose regulation (1). The appearance of lymphocytic infiltration is not restricted to the pancreas, however, and is also observed in both submandibular and lacrimal (but not parotid) glands which develop a histopathology similar to that observed in human Sjögren syndrome (2–5). Immunogenetic and lymphocyte transfer studies suggest that insulin-dependent type I diabetes and Sjögren syndrome-like sialoadenitis represent two separate autoimmune diseases taking place in the NOD mouse (6, 7). The temporal presence of leukocytes in the salivary exocrine tissue further correlates to a loss of saliva flow and tear production which is independent of the loss of blood glucose regulation (2, 8).

It is currently believed that acinar cell death observed in the exocrine tissues is a direct result of the autoimmune lymphocytic attack targeting the glands (9). Analyses of lymphocyte populations infiltrating the exocrine tissues has revealed a similar profile to that seen in the NOD islet and Sjögren syndrome with a predominance of $CD4^+$ T cells and significantly fewer $CD8^+$ T cells or B cells (10, 11). Again, consistent with the disease, cytokine profile analyses revealed temporal increases in effector cytokines tumor necrosis factor α, interferon γ, and inducible nitric oxide synthase (C.P.R., H. Yamamoto, J. Cornelius, M.G.H.-B., and A.B.P., unpublished data). *In vivo* and *in vitro* studies have implicated these effector cytokines as potential mediators of cell death through apoptotic pathways (12–14).

Our recent studies in the NOD-*scid* mouse have revealed that acinar cell death in the submandibular gland is independent of the presence of lymphocytes. The NOD-*scid* mouse contains a homozygous mutation at the severe combined immunodeficiency locus backcrossed onto the NOD genetic background, resulting in the functional loss of both T and B lymphocytes (15). Biochemical analysis of protein synthesis indicated the aberrant synthesis and processing of parotid secretory protein (PSP) in the submandibular gland of NOD-*scid* mice correlated to the time of appearance of lymphocytes in the parental NOD mouse (10, 15). In addition, histological changes in the submandibular gland were evident between 10 and 20 weeks of age, a time at which there is a loss of acinar cells and a corresponding increase in ductal cell populations. The temporal loss of acinar cells is reminiscent of the process of programmed cell death despite the absence of autoreactive lymphocytes in the tissue.

Since programmed cell death appears independently of lymphocyte infiltration of the submandibular gland, we investigated the potential activation of apoptotic proteases in the saliva and salivary gland lysates of NOD mice. The best characterized of the apoptotic cysteine proteases is the interleukin 1β converting enzyme (ICE). ICE is required for induction of apoptosis through the CD95 (FAS) signaling pathway and is necessary for the activation of interleukin 1 through cleavage of its peptide precursor (16, 17). In this study, we have found increased levels of cysteine proteases by both an *in vitro* activity assay and Western blot analyses of NOD and NOD-*scid*, as compared with BALB/c and young NOD mice. However, the ability of saliva and both submandibular and parotid gland lysates to generate the aberrant NOD PSP cleavage product suggests that additional cellular proteases are activated in these tissues as well.

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Abbreviations: NOD, nonobese diabetic; PSP, parotid secretory protein; ICE, interleukin 1β converting enzyme; BAPNA, sodium benzoyl-dl-arginine-*p*-nitroanilide.

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MATERIALS AND METHODS

Materials. BALB/c and NOD/Uf mice were bred and maintained under specially pathogen free (SPF) conditions in the mouse facility of the Department of Pathology and Laboratory Medicine (University of Florida, Gainesville). NOD-*scid* mice were purchased from The Jackson Laboratories. Both male and female mice at ages 8 weeks and 20 weeks of age were used. NOD mice were routinely tested for blood glucose levels using Chemstrip bG reagent strips (Boehringer Mannheim). Consecutive elevated fasting blood glucose levels >240 mg/dl were considered onset of diabetes, after which the mice were maintained on daily insulin injections (2). NOD mice at 20 weeks of age were separated into diabetic and prediabetic groups for protease assays.

Antibody for rat cystatin (18) was a generous gift from Gurrinder Bedi (Magainin Pharmaceuticals, Plymouth Meeting, PA). D,L-Isoproterenol, pilocarpine, phenylmethylsulfonyl fluoride, DTT, dimethyl sulfoxide, papain, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin, and sodium benzoyl-dl-arginine-*p*-nitroanilide (BAPNA) were obtained from Sigma. Reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad.

Saliva Collection and Preparation of Gland Lysates. Saliva was collected from control and experimental groups of mice following stimulation of secretion using isoproterenol (0.20 mg/100 g body weight) and pilocarpine (0.05 mg/100 g body weight) dissolved in saline. The secretagogue cocktail was injected (0.1 ml volume) i.p., with saliva subsequently collected from the oral cavity by micropipet and placed into chilled 1.5 ml microfuge tubes (2). Saliva samples were collected from groups of six mice and then frozen at −80°C until analyzed for temporal changes by enzyme assay, SDS/polyacrylamide gels, and Western blotting.

Parotid and submandibular glands were excised from mice killed by cervical dislocation. Each gland was separated from connective tissue, fat, and lymph nodes, then homogenized in 10 mM Tris buffer (pH 7.4) and immediately frozen at −80°C. Protein assays of both saliva and gland lysates were performed using the method of Bradford (19) with BSA as the standard.

Cysteine Protease Activity. Protease activity in saliva and gland lysates were determined using a standard protease assay as described (20, 21). The assay relies on the cleavage of the chromagenic reagent, BAPNA. The incubation buffer consisted of 25 μl 100 mM BAPNA in dimethyl sulfoxide, 10 μl unknown sample, 190 μl phenylmethylsulfonyl fluoride buffer consisting of 0.2 mg/ml DTT, 0.5 mg/ml Na_2EDTA, and 1.0 mM phenylmethylsulfonyl fluoride in 100 mM phosphate buffer (pH 6.0). Experimental samples, as well as a dilution profile of papain were incubated at 37°C for 1 hr. The reactions were terminated by the addition of 25 μl of glacial acetic acid, adjusted to 1.0 ml with ddH_2O, and the optical density determined at OD_{405} nm to determine the amount of *p*-nitroaniline released. A standard curve was generated from the papain. All values were expressed for the mean ± standard error for all samples performed in duplicate on three separate occasions.

PAGE and Western Blot Analysis. Total salivary proteins (15 μg/well) or gland lysates (50 μg/well) were subjected to electrophoretic separation on a 1.5-mm-thick 10% or 12% SDS/polyacrylamide gel using the modified Tris-glycine system of Pugsley and Schnaitman (22). Following electrophoresis, the proteins were transferred for 2 hr at 70 V to Immobilon-P membranes (Millipore) for Western blotting analysis (23). Polyclonal antibodies specific for murine ICE and Nedd-2 (Santa Cruz Biotechnology), and Apopain/CPP 32 (Upstate Biotechnology, Lake Placid, NY) were used to detected specific apoptotic proteases at dilutions specified by the manufacturer. Polyclonal rabbit anti-rat cystatin (1:500 dilution) was incubated with each membrane for 12 hr at 25°C. The blocking buffer consisted of 3% nonfat dry milk and 3% BSA in Tris-buffered saline. Saliva from chronically isoproterenol-treated rats was used as positive controls for assessing the performance of the anti-cystatin antibody, while preincubation of the anti-cysteine protease antibodies with the specific peptide antigen served to determine the specificity of the antibody–antigen reactions. Following three 10-min washes, the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin and exposed to substrate as described (15).

Proteolytic Digestion of Radiolabeled PSP. A mixture of saliva (10 μl) or salivary gland lysates (30 μg total protein) and 10^4 cpm of [^{125}I]PSP were incubated in a 50 μl total volume for 4–6 hr at 37°C. The digestion was stopped by the addition of SDS/PAGE sample buffer and heating to 100°C for 5 min prior to electrophoretic separation on a 12% SDS/polyacrylamide gel as described above. The gels were dried and exposed to Kodak XAR-5 x-ray film for 48 hr.

Radiolabeling of PSP was performed as described (24). Briefly, PSP was purified from BALB/c saliva using a one-step procedure by electrophoretic separation on a 3-mm preparative gel. Molecular weight standards and 0.5 cm of the sample well were removed and transferred to Immobilon-P membrane for Western blot detection of PSP using a polyclonal rabbit anti-mouse PSP antibody supplied by William Ball (Howard University, Washington, DC). Following detection, the same area of the acrylamide gel was cut from the surrounding gel and extracted in PBS containing 0.02% NaN_3 and 0.2% SDS. The extracted protein was dialyzed against ddH_2O and lyophilized to concentrate the pure PSP. Pure PSP was radiolabeled using chloramine-T and Na^{125}I obtained from Amersham. Iodinated protein was purified from contaminants by molecular sieve chromatography on Sephadex G-75 obtained from Pharmacia.

Statistical Analysis. All measures of variance are given as standard deviations of the mean. Tests of significance for differences between independent means were performed with the unpaired Student's *t* test. Results in which $P < 0.05$ were considered significant.

RESULTS

Changes in cysteine protease activity in the saliva of NOD and BALB/c control mice was assessed to correlate changes in the histology with potential apoptosis of acinar cells as observed previously (15). As presented in Table 1, there was an approximate 3-fold increase in cysteine protease activity in the saliva of 20-week-old prediabetic and diabetic NOD mice when compared with age-matched BALB/c controls or 8-week-old NOD animals ($P < 0.01$). In NOD-*scid* mice, this increase in proteolytic activity was even greater (67.5 vs. 16.7; $P < 0.005$). Saliva concentrations of cysteine protease activity ranging between 14–17 μg/ml saliva were measured in BALB/c, prediabetic NOD, and NOD-*scid* mice 8 weeks of age. The same trends in relative protease activity in saliva did not vary greatly if proteolysis was calculated relative to constant protein rather than to constant volume (Table 1). There were no differences in the levels of cysteine protease activity between 8 and 20 weeks of age in BALB/c animals or between male and female mice ($P > 0.05$).

To further define the source of the increased proteolytic activity in saliva, both parotid and submandibular gland lysates were prepared from 20-week-old mice. Parotid and lacrimal gland lysates from BALB/c, prediabetic and diabetic NOD as well as NOD-*scid* mice showed similar levels of proteolytic activity, ≈15 μg protease/mg total gland lysate protein (Fig. 1; $P > 0.05$). In contrast, gland lysates prepared from the submandibular glands of older mice revealed an increased cysteine protease activity in prediabetic NOD mice that was even greater with onset of diabetes (37.3 vs. 78.8 μg pro-

Table 1. Cysteine protease activity in saliva

	Activity,* μg/ml		Activity, μg/mg protein	
	8 weeks	20 weeks	8 weeks	20 weeks
BALB/c	15.2 ± 2.6	16.7 ± 2.7	15.9 ± 2.7	12.8 ± 2.1
NOD prediabetic mice	14.8 ± 1.9	45.8 ± 5.3	10.3 ± 1.9	39.2 ± 4.8
NOD diabetic mice	NA†	40.3 ± 6.8	NA†	39.5 ± 3.7
NOD-*scid*	12.1 ± 3.2‡	67.5 ± 12.3	13.6 ± 2.2‡	67.0 ± 4.7

*All values expressed as the mean ± standard error for $n = 6$ animals/group. The assays were performed in duplicate on three separate occasions.
†NA, not available due to the later age of diabetes onset in the University of Florida NOD colony.
‡$n = 2$.

tease/mg gland protein, respectively; $P < 0.01$). In NOD-*scid* submandibular gland lysates, proteolytic activity proved to be the greatest, correlating with previous histological analyses suggesting loss of acinar cells through cell death (15).

Detection of Elevated ICE and Nedd-2 and Apopain/CPP 32 in NOD Saliva and Gland Lysates. Saliva and gland lysates were evaluated by Western blot analysis to confirm the increased activity of apoptotic cysteine proteases in NOD mice. As presented in Fig. 2, whole saliva possessed an increase in immunoreactive material for ICE, Nedd-2, and Apopain/CPP 32 in older NOD and NOD-*scid* mice. Potential pro-enzyme forms (35–50 kDa) were detected in saliva from 8 and 20 weeks NOD as well as the NOD-*scid* animals, but not in BALB/c mice. The active subunits detected by the antibodies were: ICE (p10), Nedd-2 (p12) and Apopain (p12) (25–27). Thus, the different mobilities of the proteins recognized in the gel could be nondissociated complexes of the active apoptotic protease subunits or digestion products from interaction with proteases from saliva. The antibodies failed to react with material in gland lysates, potentially due to their low concentration in total protein preparations. As a control, for the proper identification of cysteine proteases in saliva, the same preparations were reacted with primary antibody preincubated with the peptide antigen supplied by the manufacturers. Under these conditions, only the highest molecular weight band (≅55 kDa) was detected with the addition of secondary antibody and chromogenic substrate.

Determination of Cystatin Levels by Western Blot Analysis. Cystatins are a series of cellular proteins produced by various tissues to limit proteolytic activity in response to injury (21). Synthesis of these proteins can be induced in salivary glands by chronic treatment with the β-adrenergic agonist, isoproterenol (18). Saliva was analyzed for the levels of cystatin by Western blotting using a polyclonal antibody to rat cystatin. As presented in Fig. 3, a protein of ≈15 kDa that migrated similarly to the cystatin protein present in whole rat saliva isolated following chronic isoproterenol treatment, was observed in each saliva tested. There were no differences in the levels of cystatin detected in saliva from BALB/c or NOD mice irrespective of age. Interestingly, chronic treatment of mice with isoproterenol appeared to decrease the levels of cystatin present (Fig. 3). This was reflected in a BAPNA digestion assay where proteolytic activity increased from 14 to 31 μg protease/ml and 43 to 51 μg protease/ml in BALB/c and prediabetic NOD mice, respectively ($P < 0.05$).

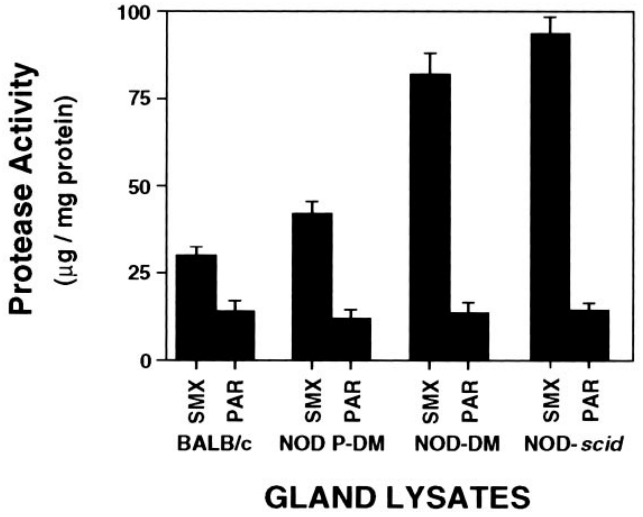

FIG. 1. Histogram of cysteine protease activity in salivary gland lysates. Parotid (PAR) and submandibular (SMX) gland lysates were incubated with chromagenic substrate, BAPNA, for 60 min at 37°C (P-DM and DM, prediabetic and diabetic, respectively). Standard curve was generated by linear regression analysis of papain digestion of BAPNA. All values represent the mean ± SE performed in duplicate on three separate occasions.

Generation of the Aberrantly Processed 17-kDa PSP Isoform. Previous reports from our laboratory have shown that NOD and NOD-*scid* mice abnormally express PSP in their submandibular glands and that with increasing age this PSP is aberrantly processed through enzymatic digestion in their submandibular glands (15, 24). Using radiolabeled PSP purified from BALB/c saliva, we assessed the ability of saliva and gland lysates to enzymatically digest PSP to the aberrant isoform. Incubation of BALB/c-derived ^{125}I-labeled PSP with NOD saliva resulted in the digestion of the 20.5-kDa PSP isoform to the 17.0-kDa PSP within 4–8 hr incubation at 37°C. Incubation for more than 8 hr caused a complete degradation of the radiolabeled PSP (data not shown). Incubation of PSP with BALB/c saliva or submandibular gland lysates failed to generate the 17.0 kDa PSP isoform (Fig. 4), and there was no evidence for partial degradation of PSP by incubation of the protein at 37°C in PBS. Also shown in Fig. 4, submandibular gland lysates from both 20-week-old prediabetic and diabetic NOD mice were able to cleave the BALB/c PSP to the aberrant 17.0 kDa isoform. Although the parotid glands from NOD mice did not show an alteration in cysteine protease activity, parotid gland lysates prepared from diabetic NOD animals could generate enzymatically cleaved PSP (Fig. 4).

DISCUSSION

The observations of Sjögren syndrome-like histopathology in the NOD mouse has led to the detailed discovery of an accompanied exocrine gland dysfunction (2, 3, 8). Temporal loss of secretory function in the salivary glands correlates with the appearance of lymphocytic foci in the submandibular glands, synthesis of proinflammatory and effector cytokines, and the presence of autoantibodies to ductal and acinar cell components of the tissues. While these autoantibodies and cytokines may play a role in effecting the loss of saliva flow and tear production, their presence may be secondary to glandular defects present in the NOD genetic background (15). Using NOD-*scid* mice, we have determined that, despite normal saliva flow and tear production, histological and biochemical changes in salivary gland architecture and secretory products

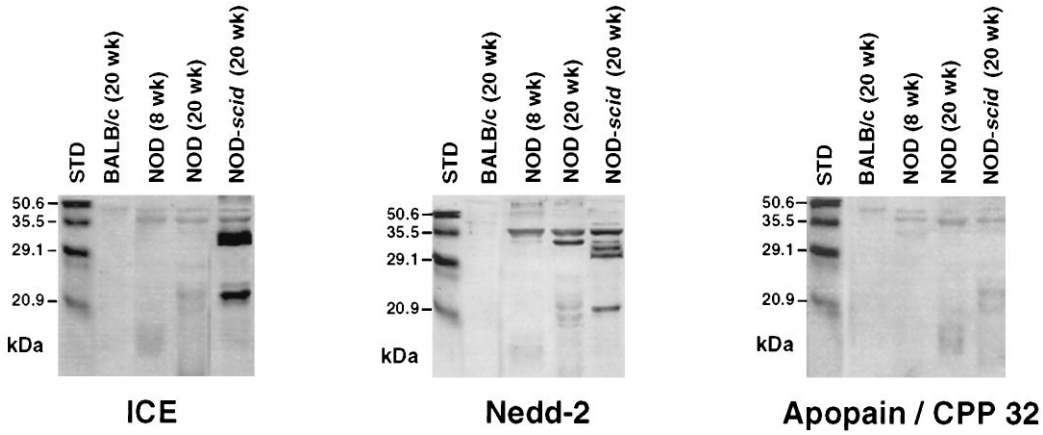

FIG. 2. Western blot analysis of saliva for the presence of apoptotic proteases. Whole saliva (15 μg) was separated on a 12% SDS/PAGE and evaluated for the presence of murine ICE, Nedd-2, and Apopain/CPP 32 using rabbit polyclonal antibodies. The fidelity of the antibody reaction was established by preincubation of the antibodies with the peptide antigen (1 μg/ml) prior to reaction with the nitrocellulose membrane. Cleavage of the ICE p45 pro-enzyme generates p20 and p10 active subunits. Proteolytic cleavage of Nedd-2 the p50 pro-enzyme generates the p20 and p12 active subunits, cleavage of the Apopain/CPP 32 p35 pro-enzyme produces the p17 and p12 subunits of the mature protease. Prestained molecular weight standards (Bio-Rad) are as follows: ovalbumin, 50,600 Da; carbonic anhydrase 35,500 Da; soybean trypsin inhibitor, 29,100 Da; and lysozyme, 20,900 Da.

occur in the absence of an active immune system (15). The most prominent change in the glandular structure was the striking age-related loss of submandibular gland acinar cells and the apparent hyperproliferation of ductal cells. In addition, the most striking biochemical change was the *de novo* synthesis of PSP in the submandibular gland and its aberrant proteolytic processing in both the parotid and submandibular glands (15, 24). Both of these observations in NOD-*scid* mice occur at the time at which the histological appearance of lymphocytic foci are detected in the parental NOD strain.

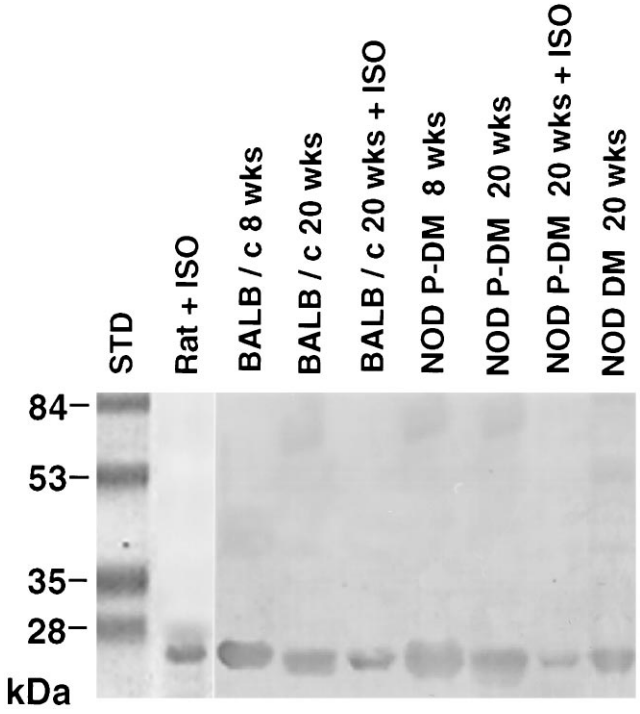

FIG. 3. Western blot analysis of saliva for the presence of the cysteine protease inhibitor, cystatin. Whole saliva (15 μg) was examined on a 12% SDS/PAGE gel using a rabbit-polyclonal anti-rat cystatin. A chronically treated isoproterenol rat whole saliva was included as a positive control. Isoproterenol treatment of mice was twice i.p. daily for 3 days. P-DM, prediabetic NOD; DM, diabetic NOD. Molecular weight standards are as follows: bovine serum albumin, 84,000 Da; ovalbumin, 54,000 Da; carbonic anhydrase, 35,000 Da; and soybean trypsin inhibitor, 28,000 Da.

Cellular homeostasis depends on regulated cell proliferation coupled to cell death. Extracellular signaling molecules are capable of regulating glandular cell populations through a series of intracellular events termed programmed cell death or apoptosis (17, 28). The cytokine tumor necrosis factor and the related protein CD95 (FAS) are immune system molecules capable of triggering apoptosis. Programmed cell death is mediated intracellularly by a proteolytic cascade involving members of the cysteine protease family which cleave important cellular proteins including pro-enzymes of other members of this class of proteases (29). A prototype protein of the cysteine proteases is ICE which has been characterized as the activator of the cytokine interleukin 1β through cleavage of its precursor at Asp-116/Ala-117 (17). All members of the ICE-related cysteine protease family cleave their substrates after an aspartate residue followed by a small amino acid residue which is important for substrate consensus recognition (29). Therefore, high levels of cysteine protease activity may be indicative of both the activation of apoptotic mechanisms as well as the processing of proinflammatory cytokine precursors.

In the present study, we have found that NOD and NOD-*scid* mice had high concentrations of cysteine protease activity in their saliva and submandibular gland lysates, consistent with the histological observations suggestive of submandibular acinar cell death. However, the parotid glands from NOD mice, which do not show evidence of a strong autoimmune attack, possessed similar levels of enzyme activity as that seen in tissue from BALB/c control mice. The increased cysteine protease activity in the submandibular glands correlated with the time at which autoimmune leukocytes generally appear in the glands and subsequently display secretory dysfunction. The greatest level of protease activity was present in the NOD-*scid* mice despite the absence of functional B and T lymphocytes. Autoimmune lymphocytes are not, therefore, the main underlying factor for increased acinar cell turnover. The increased cysteine protease activity in NOD lysates did not appear to be the result of a loss in the control of synthesis of the specific inhibitor, cystatin, as evidenced by similar saliva protein levels in BALB/c and NOD mice. Thus, the detection of cysteine protease activity in the submandibular gland is consistent with the concept that NOD mice possess a genetic predisposition for glandular loss of function leading to activation of the

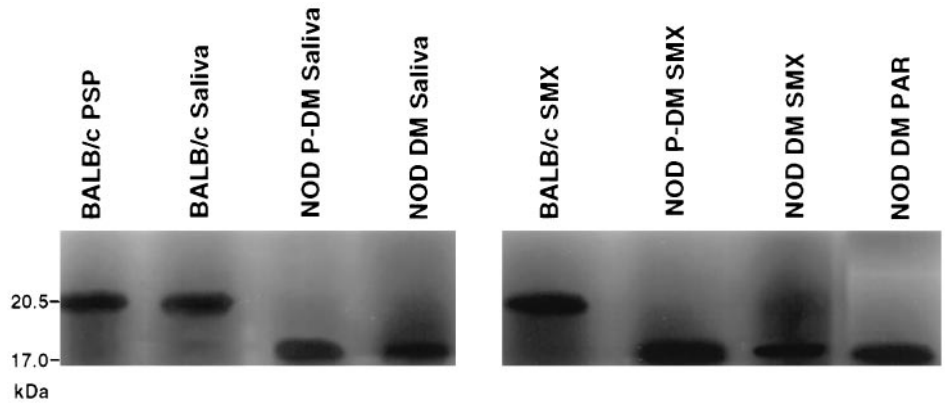

FIG. 4. Autoradiogram of differential PSP migration following incubation with saliva or salivary gland lysates. [^{125}I]PSP (10^4 cpm) purified from BALB/c saliva was incubated for 4–6 hr at 37°C with whole saliva, submandibular (SMX) lysates, or parotid (PAR) lysates from BALB/c or NOD mice. NOD P-DM, prediabetic NOD; NOD DM, diabetic NOD. Radiolabeled BALB/c PSP (lane 1) incubated in PBS served as control migration.

apoptotic pathway and subsequent activation of the immune system (15).

While parotid glands of aging NOD mice are able to generate the aberrant PSP 17.0-kDa isoform (15), levels of cysteine protease activity are similar to that of BALB/c animals. The further observation that the unique cleavage site of PSP (Asn-26/Leu-27) is not an amino acid consensus sequence for cysteine proteases, suggests that aberrant proteolytic processing of PSP in NOD mice is independent of the activation of this class of enzymes. Recent observations in our laboratory have now shown increased matrix metalloproteinase activity in the parotid and submandibular glands of NOD mice (C.P.R., S.Y., A.B.P., and M.G.H.-B., unpublished observations). Therefore, other proteases may be abnormally expressed in the salivary glands of these animals as well. Database searches of cellular proteases using this protein consensus sequence failed to identify a potential candidate responsible for the generation of the 17.0-kDa PSP isoform.

Considered as a whole, our studies are painting a complex picture as to the physiological state of the salivary glands of NOD mice. Reports (30, 31) indicate that PSP is synthesized in the neonatal submandibular gland acinar cells of normal mice up to 5 days of age after which time synthesis ceases, while synthesis in the parotid gland continues. In the NOD mouse, the reappearance of transcripts and translated PSP in the submandibular gland suggest that there is a breakdown in cell differentiation and re-expression of developmental proteins. This loss of developmental control must extend to the parotid gland with the subsequent abnormal processing of PSP in both glands dependent on an uncharacterized proteolytic activity. The more extensive glandular disruption of the submandibular gland could potentially activate programmed cell death in the acinar cells leading to the observed histopathology seen in the NOD-*scid* background. The resulting cell death may then contribute to the autoimmune pathology through the activation of immune system activators and effectors such as cytokines and the generation of autoantibodies which exacerbate the glandular dysfunction leading to loss of secretory function. The end result of this process on exocrine tissues would be clinical presentations of xerostomia and xerophthalmia.

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