THE SALIVARY GLANDS IN SJÖGREN'S SYNDROME:

PATHOGENETIC ASPECTS OF THE INITIATION OF SIALOADENITIS

DE SPEEKSELKLIEREN IN HET SYNDROOM VAN SJÖGREN:

PATHOGENETISCHE ASPECTEN VAN DE INITIATIE VAN SIALOADENITIS

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Chapter 1.1

SJÖGREN'S SYNDROME: AN OVERVIEW OF CLINICAL MANIFESTATIONS, THERAPEUTIC POSSIBILITIES, AND CURRENT KNOWLEDGE OF PATHOGENETIC MECHANISMS

Introduction

Sjögren's syndrome is a chronic inflammatory disorder with autoimmune etiology, affecting primarily the salivary and lacrimal glands. In these glands, focal lymphocytic infiltrates develop. This is accompanied by decreased production of saliva and tears, resulting in patients complaining of dry eyes and a dry mouth (1, 2). First reports in which the combination of a dry mouth and dry eyes was described date from late 19th and early 20th century (3-6). In 1933, Henrik Sjögren, a Swedish ophthalmologist, described clinical and histological findings in a group of 19 women with xerostomia and keratoconjunctivitis sicca (KCS), dry mouth and dry eyes, thirteen of which also suffered from chronic arthritis (7). At present, it has become evident that, although the presenting symptoms of Sjögren's syndrome are usually dry eyes and/or dry mouth, almost every organ in the body can be affected by the disease process. In this chapter an overview is given of the criteria that are used to diagnose Sjögren's syndrome and of the clinical manifestations of the disease. Furthermore, possibilities with regard to treatment of patients and factors that are thought to contribute to the pathogenesis of Sjögren's syndrome are discussed.

Diagnostic criteria and prevalence

At present, there is no single, universally accepted diagnostic set of criteria that is used to diagnose Sjögren's syndrome worldwide. Seven published sets of criteria which differ in sensitivity and specificity have been proposed for the diagnosis of Sjögren's syndrome (8). Criteria that are used most frequently are the Copenhagen criteria, the criteria proposed by Fox *et al*, and the European criteria (9-11). The criteria, proposed by the European Community Study Group on Diagnostic Criteria for Sjögren's syndrome in 1993, and assessed in 1996, were demonstrated to have a high sensitivity and specificity (Table 1) (11, 12). Differences between the sets of criteria may lead to variations among the patient populations that are studied by different research groups, which should be kept in mind when results of reported studies are compared.

The prevalence of Sjögren's syndrome has been estimated to be 1% of the general population, using the European Classification Criteria (Table 1) (12). In a population-based, cross sectional study among adults in the United Kingdom, the prevalence of Sjögren's syndrome according to modified European Classification Criteria was 3-4%. Lip biopsies were not performed in this study because this was considered unethical in a community survey, but patients had to fulfill at least four of the remaining criteria (13). Sjögren's syndrome predominantly affects women, with a female: male ratio of 9:1 and a peak incidence around the fifth decade of life, although there is growing awareness that Sjögren's syndrome can also affect young adults, adolescents, and even children. The presenting symptom of juvenile Sjögren's syndrome is most often parotitis, whereas sicca symptoms usually develop later in the disease process (14, 15). Although the initial manifestations of Sjögren's syndrome may differ between adults and children, complications of the disease are comparable (16, 17). Sjögren's syndrome can occur as an isolated disorder or in addition to another autoimmune

disease, such as rheumatoid arthritis or systemic lupus erythematosus (SLE), and is classified as primary or secondary Sjögren's syndrome, respectively (Table 1) (12).

Table 1. European Classification Criteria for Sjögren's syndrome

I. Ocular symptoms:

A positive response to at least one of the three selected questions:

1. Have you had daily, persistent, troublesome dry eyes for more than three months?
2. Do you have a recurrent sensation of sand or gravel in the eyes?
3. Do you use tear substitutes more than three times a day?

II. Oral symptoms:

A positive response to at least one of the three selected questions:

1. Have you had a daily feeling of dry mouth for more than three months?
2. Have you had recurrent or persistently swollen salivary glands as an adult?
3. Do you frequently drink liquids to aid in swallowing dry foods?

III. Ocular signs:

Objective evidence of ocular involvement defined as a positive result in at least one of the following two tests:

1. Schirmer's test (≤ 5 mm in 5 minutes) in patients ≤ 60 years
2. Rose bengal score (≥ 4 according to Van Bijsterveld scoring system)

IV. Histopathology:

A focus score ≥ 1 in a minor salivary gland biopsy (a focus is defined as an agglomerate of at least 50 mononuclear cells; the focus score is defined by the number of foci in 4 mm^2 of glandular tissue)

V. Salivary gland involvement:

Objective evidence of salivary gland involvement defined by a positive result in at least one of the three diagnostic tests:

1. Salivary scintigraphy
2. Parotid sialography
3. Unstimulated salivary flow (≤ 1.5 ml in 15 minutes) in patients ≤ 60 years

VI. Autoantibodies:

Presence in the serum of the following antibodies:

Antibodies to Ro (SSA) or La (SSB), or both

Primary Sjögren's syndrome: 4 out of these 6 items.

Secondary Sjögren's syndrome: A diagnosis of a connective tissue disease on the basis of well-defined and commonly accepted criteria, and a positive response to item 1 or 2, plus a positive response to at least 2 items among items 3, 4, and 5.

Exclusion criteria: Pre-existing lymphoma, AIDS, sarcoidosis, or graft-versus-host disease, sialoadenosis, use of antidepressant and anti-hypertensive drugs, neuroleptics, parasympatholytic drugs.

Clinical manifestations

Patients with Sjögren's syndrome may suffer from complaints directly related to dryness of the eyes and mouth, from generalized complaints such as fatigue and depression, as well as from disease manifestations due to involvement of other organs. The decreased production of saliva can lead to oral soreness, difficulty with mastication, loss of taste, recurrent oral infections, and severe dental caries. The latter two are due to loss of the bactericidal effect of salivary enzymes (18). Early tooth-loss, which has been described in a significant percentage of patients, correlated significantly with the degree of lymphocytic infiltration of

the salivary glands, but not with any other manifestation of oral involvement (19). Due to decreased tear secretion, patients may complain of foreign body sensation in the eyes, red eyes, itch and even diminished sharpness of sight (18).

A significant percentage of patients with Sjögren's syndrome suffers from chronic fatigue. The seriousness of this problem was recently demonstrated in a study in which Sjögren's patients were asked to complete a questionnaire covering different aspects of fatigue. Fifty percent of patients syndrome were burdened by fatigue. Some aspects could be related to depressive symptoms (mental fatigue, reduced motivation), whereas physical aspects of fatigue were suggested to reflect disease activity (20). In a community-based survey in the UK, patients that were diagnosed as having Sjögren's syndrome also suffered from higher levels of fatigue and depression as compared to those without this diagnosis (13). Sleep disturbances, including difficulties falling asleep, as well as frequent awakenings during the night, were frequently observed among patients with Sjögren's syndrome. Patients themselves identified the disturbed sleeping pattern as a strong contributing factor to their fatigue (21). Disturbed initiation of sleep may be due to 'racing thoughts' and anxiety, whereas increased need to drink during the night may contribute to the frequent awakenings. Depression and anxiety are common symptoms in patients with Sjögren's syndrome. Various degrees of anxiety and depression were reported in 48% and 32% of patients with Sjögren's syndrome, respectively. On the other hand, patients with rheumatoid arthritis reported these symptoms to the same extent as healthy controls, suggesting that mechanisms other than musculoskeletal pains must underlie these complaints in Sjögren's syndrome (22). In patients with Sjögren's syndrome, hypofunction of the hypothalamic-pituitary-adrenal axis has been demonstrated. Basal, as well as stimulated ACTH and cortisol levels were significantly decreased in patients as compared with controls (23). This has been suggested to contribute to fatigue and mood disorders.

In addition to the salivary and lacrimal glands, kidneys, bladder, stomach, liver, exocrine pancreas, thyroid gland, lungs, heart, blood vessels, and skin may be affected in patients with Sjögren's syndrome. The most common clinical manifestation of renal involvement is an inability of the distal renal tubule to secrete hydrogen ions, which may lead to complete or incomplete distal renal tubular acidosis, as well as mild proteinuria (24, 25). Tubulointerstitial nephritis is the predominant kidney lesion in patients with Sjögren's syndrome (26). Renal tubular acidosis was mainly observed in patients with high levels of anti-SS-A/Ro or anti-SS-B/La antibodies and extensive infiltration of the minor salivary gland (MSG), but no association was found between the MSG and the renal focus scores (24, 26). Sjögren's syndrome, or the oral or ocular hallmark of the disease, was reported in a high percentage of patients with interstitial cystitis, a nonbacterial inflammatory disease of the bladder. This suggests that interstitial cystitis can occur in association with Sjögren's syndrome and may form part of the clinical spectrum of this disease (27).

Gastric manifestations of Sjögren's syndrome include nausea, pain in the stomach, and chronic atrophic gastritis (28). Biopsy specimens revealed chronic inflammation with mononuclear cell infiltrates and/or glandular atrophy (29). Abnormal liver function tests have

been reported in patients with Sjögren's syndrome, but the frequency varied among different studies (30, 31). The associated liver diseases include primary biliary cirrhosis (PBC) and autoimmune chronic active hepatitis. Among patients with PBC, a high prevalence of Sjögren's syndrome has been demonstrated (32). When monoclonal antibodies directed to an autoantigen in PBC (PDC-E2) were used in immunohistochemical stainings on salivary glands of patients with PBC, intense staining of the ductal epithelial cells, comparable to that observed in the biliary epithelium, was demonstrated in a high proportion of patients. However, this was independent of the presence of clinical or histologic features of Sjögren's syndrome (33).

Elevated pancreatic enzyme levels have been reported in a high percentage of patients with Sjögren's syndrome, PBC, and patients with both diseases (34). A link between chronic idiopathic pancreatitis and Sjögren's syndrome was suggested following the observation that peripheral blood lymphocytes of 55% of patients with Sjögren's syndrome and of 33% of patients with chronic pancreatitis showed a proliferative response to a partially purified pancreatic antigen (35). Furthermore, in serum of 27% of patients with Sjögren's syndrome, autoantibodies directed to this pancreatic antigen were detected (36). However, the pancreatic antigen used in these experiments was recognized by a monoclonal antibody that also reacts with an antigenic determinant expressed by the duct cells of other exocrine organs, including the salivary glands, bile ducts, and distal renal tubules. This implies that the pancreatic antigen used in these experiments, or an antigenic determinant present within the antigen, is also expressed by salivary gland epithelial cells, and that the pancreatic origin of the antigen is probably of minor importance. Still, regardless of the primary origin of the antigen or the role of the autoantibodies in the pathogenesis of both diseases, an association between Sjögren's syndrome and idiopathic chronic pancreatitis was demonstrated.

Thyroid disease and thyroid dysfunction have been reported in a high percentage of patients with Sjögren's syndrome (37-40). This included autoimmune thyroid disease (ATD), in which autoantibodies directed to thyroglobulin, thyroid peroxidase, or thyroid hormones were present in the serum of patients, as well as non-autoimmune thyroid disease (NATD) (37, 40). This resulted most frequently in subclinical hypothyroidism in Sjögren's patients with ATD, whereas NATD patients with Sjögren's syndrome mainly suffered from hyperthyroidism. However, although the prevalence of thyroid disease was high in patients with Sjögren's syndrome, it was not significantly different compared with age- and sex-matched controls (36% vs. 27%) (40). When features of Sjögren's syndrome were examined in a group of patients with ATD, keratoconjunctivitis sicca, xerostomia and a positive labial gland biopsy were found in 24% of patients. No significant differences were found between patients with Graves' disease or Hashimoto's thyroiditis. Although the underlying pathogenetic process of sialoadenitis (lymphocytic infiltration of the salivary glands) may vary among patients with ATD and Sjögren's syndrome, the immunopathological picture of sialoadenitis in both patients groups was similar (41).

Lung involvement was common in a group of 61 patients with primary Sjögren's syndrome. Histopathologically, submucosal mononuclear infiltrates were present in the

bronchial tree, mostly in the small bronchioles. However, most patients only suffered from dry cough without specific clinical findings, and clinical airway obstruction was observed in only 10% of patients (42). Another histopathological finding was the presence of an increased number of $CD4^+$ T cells within the bronchial mucosa, both in patients with primary and secondary Sjögren's syndrome (43). Dyspnea on exertion and recurrent bronchitis have been reported in a significant percentage of Sjögren's patients, whereas no abnormalities were observed on chest radiographs (44). Bronchial hyperresponsiveness is another frequent observation in patients with Sjögren's syndrome (45, 46). In a ten year follow-up study on pulmonary function of Sjögren's patients, it was found that, although pulmonary complications can be a significant threat to health and account for considerable morbidity, most patients did not develop progressive lung disease (47). Cardiac manifestations of Sjögren's syndrome are rare: in a study among 54 patients with definite primary Sjögren's syndrome, only one patient exhibited acute exudative pericarditis. Clinically silent changes may however be common, since an echodense pericardium, which can be a consequence of symptom free pericarditis, has been reported in 33% of patients (48).

Vasculitis has often been described in patients with Sjögren's syndrome. In a study on 70 patients, vasculitis was evident in 9 patients. Gastrointestinal tract, skin and peripheral nerves were consistently involved by vasculitis; both small and medium-sized vessels were affected. The severity of vasculitis was demonstrated by the fact that one patient even died of vasculitis (49). Peripheral neuropathy is a common finding among patients with Sjögren's syndrome, and may be related to the vasculitis process within the peripheral nerves. It most commonly presents as a sensory neuropathy, but motor and autonomic nerves can also be affected (50). In a group of 46 patients with primary Sjögren's syndrome, peripheral neuropathy was reported in 10 patients. In 5 of these patients, neurologic involvement was the main feature of the disease, demonstrating the significance of peripheral nerve involvement (51). Neurologic disease in patients with Sjögren's syndrome (reviewed in (50)) can also affect the central, in addition to the peripheral nervous system. Central nervous system disease in patients with Sjögren's syndrome (CNS-SjS) can include movement disorders and tremors, visual loss, but may also result in psychiatric and cognitive dysfunction, and dementia. Histopathologically, mononuclear inflammatory infiltrates surrounding, and in some cases invading small blood vessels in the brain of patients with CNS-SjS can be observed. These infiltrates are often associated with micro-infarcts, suggesting that CNS-SjS is mainly the result of the vasculitic process, occurring within the CNS (50).

Lymphoma is a serious complication in primary Sjögren's syndrome. The percentage of patients developing lymphoma has been estimated between 5 and 10%, of which non-Hodgkin lymphomas (NHL) are most frequently observed (52-54). When the prevalence of Sjögren's syndrome among patients with untreated NHL was examined, 14 of 113 patients had Sjögren's syndrome according to the Greek criteria, whereas another 12 patients had a positive focus score in their minor salivary gland (55). Lymphomas that develop in patients with Sjögren's syndrome are predominantly observed in extranodal sites, and are most often identified in the salivary glands. Patients with NHL in addition to Sjögren's syndrome more

often suffered from lymphadenopathy, skin vasculitis, and peripheral nerve involvement, as compared with the general Sjögren's syndrome population (56). Although the mechanism leading to the transition of benign clusters of lymphocytes to malignant lymphomas is not known, some authors believe that rheumatoid factor producing B cells play a role in this process (57). Increased expression of anti-apoptotic molecules by lymphocytes infiltrating the salivary glands (which may contribute to the persistence of the lymphocytic infiltrates, as will be discussed later) has also been suggested to be responsible for the high prevalence of lymphoma in Sjögren's syndrome (58).

An overview of the clinical manifestations that can occur in patients with Sjögren's syndrome is given in Table 2.

Treatment

Treatment of Sjögren's syndrome involves topical therapy aimed at direct alleviation of the oral and ocular complaints or reduction of local inflammation, as well as systemic therapy. Topical therapy includes the use of fluoride, dental implants, oral hygiene, dietary counseling, as well as saliva substitutes, whereas artificial tears and topical cyclosporin have been used to treat keratoconjunctivitis sicca (59-63). Topical cyclosporin treatment of KCS resulted in maintenance of the structural integrity of the epithelium, and a reduction of activated lymphocytes within the conjunctiva of patients with KCS (61, 62).

Several anti-rheumatic drugs have been used in the systemic treatment of Sjögren's syndrome, including steroids like prednisone, and non-steroidal anti-inflammatory drugs (NSAID), for example piroxicam (reviewed in 63). Although patients receiving prednisone for six months reported a decrease in their oral dryness more frequently than patients receiving piroxicam or placebo, functional and histological parameters of Sjögren's syndrome were not significantly improved by any of these drugss (64). Another example of an anti-rheumatic drug that has been prescribed to patients is the antimalarial drug hydroxychloroquine. This drug has first been used and proved beneficial in patients with rheumatoid arthritis and SLE. Although the exact mechanism of action is not fully understood, hydroxychloroquine has been suggested to interfere with antigen processing by macrophages and other antigen presenting cells (APC), resulting in diminished activation of T lymphocytes (65). In a retrospective study on effectiveness of hydroxychloroquine among 50 patients with primary Sjögren's syndrome, significant improvement of ocular symptoms as well as improved corneal integrity was observed in over 50% of the patients. In addition, oral symptoms and objective tests for oral involvement, including salivary flow rate, were improved in the majority of patients, whereas serum IgG levels decreased. This was accompanied by decreased symptoms of fatigue and an increased feeling of well-being (66). Two other studies however reported less convincing data on improvement of subjective findings in patients with Sjögren's syndrome following treatment with hydroxychloroquine, although serum immunoglobulin concentrations were also significantly decreased (67, 68). Treatment of patients with Sjögren's syndrome with 200 mg hydroxychloroquine per day for one year resulted in decreased salivary and serum levels of IL-6 and decreased salivary hyaluronic acid

Table 2. Reported clinical manifestations related to Sjögren's syndrome

Oral complaints	-	oral soreness	(18, 19)
	-	difficulty with mastication	
	-	loss of taste	
	-	recurrent oral infections	
	-	dental caries, early dental loss	
Ocular complaints	-	foreign body sensation	(18)
	-	red eyes	
	-	itch	
	-	diminished visual acuity	
Chronic fatigue			(13, 20, 21)
Depression and anxiety			(22)
Kidney	-	renal tubular acidosis	(24-26)
	-	mild proteinuria	
	-	tubulointerstitial nephritis	
Bladder	-	interstitial cystitis	(27)
Stomach	-	nausea, pain	(28)
	-	chronic atrophic gastritis	
Liver	-	primary biliary cirrhosis	(30-32)
	-	chronic active hepatitis	
Pancreas	-	chronic idiopathic pancreatitis	(35)
Thyroid gland	-	autoimmune and nonautoimmune thyroid disease	(37, 39, 40)
	-	thyroid dysfunction	
Respiratory system	-	recurrent bronchitis	(44-46)
	-	bronchial hyperresponsiveness	
Heart	-	pericarditis	(48)
Blood vessels	-	vasculitis	(49)
Neural tissue	-	peripheral neuropathy	(50, 51)
	-	central nervous system involvement	
Lymphoma			(52-56)

levels. Despite a decrease in these inflammatory markers among all treated patients, the clinical effect was disappointing. It was suggested that the duration of the treatment in this study and in other studies that failed to demonstrate a clear beneficial effect, was not sufficient to result in improved clinical symptoms, since the effect on the salivary inflammatory component was impressive (69).

In addition to topical use of cyclosporin, the effect of systemic treatment of Sjögren's

patients with cyclosporin has also been studied. Cyclosporin A can suppress T cell proliferation by inhibition of interleukin-2 production. Although symptoms of xerostomia decreased, no objective effect on salivary and lacrimal gland function was observed. Furthermore, no consistent effect of systemic cyclosporin A treatment on the histopathological lesion in the MSG has been found. Effects that were reported include a decrease in the number of T lymphocytes, an unchanged histopathological lesion, or even an increased mean focus score (70-72). The serious side effects of systemic treatment with cyclosporin A, including nephrotoxicity and increased risk of tumor development, is a major disadvantage of this drug and requires careful monitoring of the patients.

Pilocarpine is a muscarinic cholinergic agonist that stimulates salivary and lacrimal secretion, both in healthy subjects and in patients with decreased glandular function. These effects occur shortly (15 minutes) after oral administration and maintain for at least one hour. In patients with Sjögren's syndrome, a significant increase in labial salivary gland flow as well as whole salivary flow was observed in several studies following administration of pilocarpine, whereas improvement of tearing was usually less significant (73, 74). However, in a multicenter study on 373 patients with primary and secondary Sjögren's syndrome, treated daily with oral pilocarpine for 12 weeks, global assessments of both dry mouth and dry eyes were significantly improved (75). Long-term use of pilocarpine is safe and side effects are generally mild, including increased sweating and gastrointestinal symptoms (75, 76). The beneficial effects of pilocarpine on exocrine gland function fit well with new insights on the role of anti-muscarinic receptor antibodies in diminished secretory function in Sjögren's patients (77), which will be discussed in a subsequent section. Bromhexine is another compound that, when administered systemically, may influence glandular secretion. Tear secretion was improved in a high percentage of patients as well as controls, treated with bromhexine for three weeks, whilst an amelioration of xerostomia was also reported. Side effects of bromhexine treatment were negligible (78, 79).

Autoantibodies

Patients with Sjögren's syndrome can have autoantibodies also commonly detected in the serum of patients with other systemic rheumatic diseases, such as SLE and scleroderma, as well as autoantibodies that are specific for Sjögren's syndrome and have been implicated in the pathogenesis of the disease. Serum antinuclear antibodies (ANA) are highly characteristic for systemic rheumatic diseases. The predominant ANA in Sjögren's syndrome are antibodies to the ribonucleoproteins SS-A/Ro and SS-B/La. The presence of these antibodies is regarded as one of the hallmarks of the disease. Antibodies to SS-A/Ro have been described in 50-75% of patients with Sjögren's syndrome, whereas anti-SS-B/La antibodies were found in 20-50% of patients (80, 81). Even higher frequencies of seropositive patients were reported when an ELISA was used to study the presence of anti-SS-A and anti-SS-B (82). Whereas anti-SS-A/Ro antibodies are also present in the serum of a high percentage of patients with SLE, anti-SS-B/La antibodies are closely associated with Sjögren's syndrome (83). IgG antibodies have been shown to dominate the autoantibody response to SS-A/Ro and

SS-B/La, followed by IgM and IgA (84).

The SS-A/Ro autoantigen is a ribonucleoprotein (RNP) complex, containing at least two proteins, Ro 60 kD and Ro 52 kD, which are associated with a set of small RNAs, also named human cytoplasmic RNAs (yRNA) (85-87). The 60 kD SS-A/Ro protein possesses RNA binding sequences as well as a single zinc-finger motif, whereas putative zinc-finger domains and a leucine zipper motif were identified in the amino-terminal half of the 52 kD protein (85, 86). The exact function of the Ro proteins is not known. The SS-B/La autoantigen consists of a 48 kD protein that can physically associate with the Ro/RNP particle, and serves as a termination factor for RNA polymerase III (88, 89). Antibodies to 52 kD SS-A/Ro or 48 kD SS-B/La are often present in sera that also contain antibodies to 60 kD SS-A/Ro, and are rarely found in isolation, which may indicate epitope spreading among different constituents of the RNP complex (90). Anti-SS-A/Ro positive sera can also react with a cytoplasmic constituent, in addition to a nuclear constituent. It has been suggested that SS-A/Ro binds to newly synthesized RNA in the nucleus, after which the complex is transported to the cytoplasm (91, 92). In contrast to anti-SS-A/Ro antibodies, anti-SS-B/La positive sera mainly bind to nuclear constituents, producing a nuclear speckled pattern (93). Translocation of SS-A/Ro and SS-B/La to the cell membrane has been described, but not under normal conditions. Stimuli that were shown to induce membrane translocation include UV-irradiation, virus infection (but not by all viruses), and apoptosis (94-96). The potential involvement of autoantibodies to SS-A/Ro and SS-B/La in the pathogenesis of Sjögren's syndrome was suggested following the demonstration of anti-Ro 52 kD, anti-Ro 60 kD, and anti-La autoantibody-producing cells in MSG of Sjögren's patients. A correlation was observed between the quantity and isotype distribution of autoantibodies in the serum, and the autoantibody-producing cells in the salivary glands (97). However, the precise role of anti-SS-A and anti-SS-B antibodies in the clinical picture of Sjögren's syndrome has not yet been clarified.

Maternal anti-SS-A/Ro and anti-SS-B/La antibodies have been suggested to play a role in the development of congenital heart block (CHB) in infants with neonatal lupus erythematosus (NLE). The combination of antibodies to SS-B/La and 52 kD SS-A/Ro has been shown to be significantly increased in mothers of children with NLE. Furthermore, 52 kD SS-A/Ro and 48 kD SS-B/La were found to be abundantly expressed in fetal cardiac tissues (98). The question remains how antigens, normally present within the nucleus and cytoplasm of the cell, become accessible for maternal antibodies. As mentioned before, apoptosis may induce translocation of SS-A/Ro and SS-B/La to the cell surface. It was shown that surface blebs of apoptotic keratinocytes contained both SS-A/Ro and SS-B/La (96). Evidence for the existence of this pathogenetic mechanism was provided by the demonstration that biotinylated surface proteins from apoptotic fetal cardiocytes were immunoprecipitated by antiserum recognizing SS-A/Ro and SS-B/La. Scanning electron microscopy studies revealed diffuse binding of anti-SS-A/Ro and anti-SS-B/La antiserum to surface blebs of the apoptotic cells, but not to non-apoptotic cells. Hereafter it was shown that coculture experiments of macrophages with apoptotic cardiocytes that had been incubated with anti-SS-A/Ro or anti-SS-B/La resulted in an increased production of TNF-α. It was suggested that opsonization of

apoptotic cells by maternal antibodies may change the otherwise innocent degradation products, normally produced extensively during embryogenesis and morphogenesis, into proinflammatory stimuli. This could result in permanent damage to the cardiac tissue, which has low regenerative capacity, eventually resulting in congenital heart block (99).

Rheumatoid factor (RF) autoantibodies are another example of autoantibodies that can be detected in the serum of patients with Sjögren's syndrome, but also in patients with other systemic autoimmune disorders (100). When isotype distribution of RF in the serum of patients with Sjögren's syndrome was examined, the presence of both IgA-RF and IgM-RF was revealed, whereas IgG-RF was not detected (101, 102). IgA-RF was also demonstrated in saliva samples, and when corrected for total IgA concentrations in both compartments, a relative concentration ratio higher than 1 was found, implicating local production of IgA-RF in the salivary glands of Sjögren's patients. IgM-RF levels on the other hand were measured in serum of patients in the absence of detectable levels in the saliva, suggesting that this RF isotype is mainly produced in the non-mucosal compartment (102).

Recently, the presence of antibodies to 120 kD α-fodrin was described in the serum of 41 of 43 patients with Sjögren's syndrome, whereas these autoantibodies were absent in sera from patients with rheumatoid arthritis or SLE (103). Fodrin is a major component of the cytoskeleton of most eukaryotic cells, which forms heterodimers composed of a 240 kD α-subunit and a 235 kD β-subunit (104). The 240 kD α-subunit can be cleaved by proteases that are activated in association with apoptosis, resulting in generation of a 120 kD sized α-fodrin (105, 106). In another study, the prevalence of anti-α-fodrin autoantibodies was lower, namely 78% and 60% in patients with primary and secondary Sjögren's syndrome, respectively, whereas 7% of sera from patients with SLE demonstrated binding to the recombinant protein (107). IgA anti-α-fodrin antibodies were more prevalent in the serum of patients with Sjögren's syndrome as compared with those of the IgG isotype. Furthermore, the latter were less specific for Sjögren's syndrome, as they were also detected in the serum of 5 of 12 patients with rheumatoid arthritis. From this study it was concluded that IgA rather than IgG antibodies against α-fodrin may be useful markers for Sjögren's syndrome (108). The potential contribution of 120 kD α-fodrin to the pathogenesis of Sjögren's syndrome will be discussed in a subsequent section.

Another group of serum autoantibodies specifically found in serum of patients with Sjögren's syndrome, and which have been implicated in the pathogenesis of the disease, are autoantibodies directed to the muscarinic cholinergic receptor. Muscarinic receptors of the M3 subtype are expressed on acinar cells of exocrine glands as well as on bladder and intestinal smooth muscle cells (109, 110). They mediate fluid secretion by the salivary and lacrimal glands and contraction of the bladder and intestinal smooth muscle cells following agonistic stimulation (111-114). Although M1 muscarinic receptors are also expressed by acinar cells of exocrine glands, the importance of the M3 muscarinic receptor in the process of salivary secretion was demonstrated in mutant mice lacking the M3 receptor, in which decreased salivary flow rates were measured upon injection with pilocarpine. Other effects observed in these mice were decreased pupillary constriction and decreased *in vitro* bladder

detrusor contractions (115). The latter may contribute to bladder irritability, which has been observed in a part of the patients with Sjögren's syndrome, whereas tonic pupils have also been described in patients (77).

The presence of autoantibodies against the muscarinic receptor in patients with Sjögren's syndrome was first suggested following experiments in which it was shown that the IgG fraction of serum of Sjögren's patients (SjS-IgG) could inhibit binding of the muscarinic receptor radioligand [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) to mouse or rat parotid gland muscarinic receptors (116, 117). This inhibition was due to a decreased number of binding sites available for binding by the radioligand, following incubation with SjS-IgG (116). Competition curves with selective muscarinic receptor antagonists revealed that the muscarinic receptor, recognized by SjS-IgG was of the M3 subtype. The anti-M3 muscarinic receptor antibodies, present in SjS-IgG were agonistic, since they induced a comparable effect in the parotid gland membranes as the muscarinic receptor agonist carbachol. No correlation was found between binding of SjS-IgG antibodies to the M3 muscarinic receptor and the presence of anti-SS-A/Ro or anti-SS-B/La antibodies in the serum (116). Subsequent experiments by the same group included Western blot analysis on rat lacrimal gland membranes, in which SjS-IgG was shown to contain antibodies binding to a 70 kD protein that co-migrated with the peak of labeled muscarinic receptors (118). In addition, immunofluorescence experiments on rat lacrimal glands demonstrated staining of glandular epithelial cells by SjS-IgG. The staining intensity was attenuated by preincubation of the IgG fraction with a synthetic peptide, corresponding to the second extracellular loop of the M3 muscarinic receptor, demonstrating that reactivity of the autoantibodies is primarily directed to the M3 muscarinic receptor subtype (119).

In the studies mentioned above, only serum from patients with primary Sjögren's syndrome was analyzed. Recently, the presence of anti-M3 muscarinic receptor antibodies was examined in serum of patients with primary and secondary Sjögren's syndrome, by means of a functional assay (77). Contraction of bladder smooth muscle, induced by carbachol, could be inhibited to about 50% by serum or IgG from 5 of 9 patients with primary Sjögren's syndrome and 6 of 6 patients with secondary Sjögren's syndrome. The remaining contraction could be abolished by the M3 muscarinic receptor antagonist 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP). Also the effect of endogenously derived acetylcholine on M3 muscarinic receptor mediated bladder contraction could be inhibited to 50% by SjS-serum. Although the autoantibodies in this study had antagonistic properties, which is in contrast to the results of Bacman *et al* as described above, an acute agonistic effect - contraction of bladder detrusor muscle - was observed in one-third of patients, both with primary and secondary Sjögren's syndrome. This effect declined with time, indicating receptor desensitization (77).

In addition to antibodies to the M3 muscarinic receptor, antibodies to the M1 muscarinic receptor have been detected in serum of patients with Sjögren's syndrome. Upon incubation with SjS-IgG, increased nitric oxide synthase activity was observed in rat submandibular glands. This was completely inhibited by the M3 muscarinic antagonist 4-DAMP,

and partially blocked by the selective M1 muscarinic receptor antagonist pirenzepine. Preincubation of SjS-IgG with a synthetic peptide, corresponding to the second extracellular loop of the M1 muscarinic receptor, had a similar inhibitory effect (120). In addition to anti-SS-A/Ro and anti-SS-B/La antibodies, anti-M1 muscarinic receptor antibodies have also been implicated in the pathogenesis of CHB. SjS-serum revealed a positive image in immunofluorescence studies on neonatal atria slices, which could be abrogated by preincubation with a synthetic M1 muscarinic receptor peptide (121). IgG, purified from sera of children with CHB or their mothers, inhibited binding of [^{3}H]-QNB to neonatal rat atria, and decreased contractility of neonatal atria. These effects were not observed when adult rat atria were used (122). Similar results were obtained when the effects of SjS-IgG on rat neonatal atrial membranes were examined (123). The observation that cardiac block in mothers of infants with CHB is rare may be due to the fact that, whereas the M1 muscarinic receptor is expressed by neonatal atria (in addition to the M2 muscarinic receptor), expression is absent in adult cardiac tissue (124, 125).

Histopathology

Immunohistologic studies of the focal lymphocytic infiltrates in MSG of patients with Sjögren's syndrome have revealed a predominance of T lymphocytes over B lymphocytes. The majority of T cells exhibited a T helper phenotype, and a ratio of CD4: CD8 > 2 was observed in all cases, independent of focus size (126-130). The activation antigens CD25 and HLA-DR were highly expressed on the infiltrating T cells (126, 129, 131). Several groups have studied TCR Vß usage of T lymphocytes infiltrating the MSG. These studies demonstrated limited, but not restricted TCR Vß usage, with predominant expression of Vß2, Vß8, and Vß13 (132, 133). Others found restricted TCR Vß usage only in the early stage of disease, whereas a large number of Vß families was expressed in patients with advanced lymphocytic infiltration and salivary gland fibrosis (134). Jß genes that were rearranged to the amplified Vß genes in the patients with the early stage of disease also showed restriction, suggesting a monoclonal or oligoclonal expansion of infiltrating T cells in these patients (134, 135). The polyclonal nature of infiltrating cells in the late stage of disease on the other hand may reflect secondarily recruited cells to the inflammatory environment. Interestingly, when TCR Vß genes expressed in the salivary and lacrimal glands of the same patient were analyzed, common complementarity determining region 3 (CDR3) sequences were found in both locations (136). Since the CDR3 region of the T cell receptor contacts the peptide, bound by the HLA molecule, the expression of common CDR3 sequences by T cells in the salivary and lacrimal glands suggests that some infiltrating T cells in both locations recognize the same autoantigens. In addition to T and B cells, macrophages have been detected within the infiltrates in the MSG. These cells were also present in the interstitial tissue (137). The macrophages within the focal infiltrates were found to contain calprotectin, a product with antimicrobial properties that can be released by activated macrophages. This was suggested to reflect the chronic nature of the inflammatory process (137).

Cytokine expression by infiltrating lymphocytes in the salivary glands of patients with

Sjögren's syndrome has been the subject of a large number of studies. PCR experiments on mRNA of $CD4^+$ T lymphocytes eluted from MSG of patients with Sjögren's syndrome revealed high expression of interleukin (IL)-2, interferon (IFN)-γ, and IL-10, whereas IL-4 and IL-5 mRNA were not expressed. Analysis of saliva samples of the same patients indicates that the mRNA expression is followed by the production of the corresponding cytokines (138). By *in situ* hybridization, IL-2 and IL-2R mRNA expression was demonstrated among infiltrating lymphocytes. In addition, IL-4 mRNA expression was found, but only in tissues from patients with small lymphocytic infiltrates. Other cytokines that were expressed include IL-1ß, tumor necrosis factor (TNF)-α, and IL-6, but the cellular sources of these cytokines were not identified. IFN-γ mRNA expression was only observed in 3 out of 12 biopsies, whereas IL-10 mRNA was not detected in this study, although this was studied in only four samples (139). Following isolation of $CD4^+$ T cell clones from the salivary glands of patients with Sjögren's syndrome, production of IFN-γ, IL-2, high levels of IL-10, and little IL-4 was demonstrated (140, 141). Most other studies report on the expression of cytokine genes using mRNA samples isolated from total MSG, which does not allow identification of the cell type, responsible for expression of the particular cytokine(s). However, comparison of mRNA expression levels of cytokines in salivary glands of patients with Sjögren's syndrome with those in controls revealed differences that were due to the presence of lymphocytic infiltrates and to their effect on the surrounding cells. Cytokines that were specifically expressed in total minor salivary gland samples of patients with Sjögren's syndrome, in addition to the cytokines mentioned above, include IL-12, IL-13, and IL-18 (142, 143). Transforming growth factor (TGF)-ß mRNA was also observed in MSG. In glands with a high focus score, however, the expression level was low, which may indicate that TGF-ß limits progression of the sialoadenitis (144). In contrast to the findings by Fox *et al* (138), another group reported mRNA expression of IL-4 and IL-5 in MSG of patients with Sjögren's syndrome. This correlated closely with the number of B cells in these glands, suggesting a contribution of these cytokines to the accumulation and/or expansion of B cells in the salivary glands (141). In conclusion, these studies demonstrate a predominance of Th1-like cells within the lymphocytic infiltrates in MSG of patients with Sjögren's syndrome, although expression of the Th2 cytokines IL-6 and IL-10 is also significant (Table 3). The role of the cytokines that are expressed by infiltrating lymphocytes in the pathogenesis of Sjögren's syndrome will be dealt with in a subsequent section.

Recently, the expression of chemokines has been examined in MSG of patients with Sjögren's syndrome. The large majority of infiltrating lymphocytes produced macrophage inflammatory protein (MIP)-1ß, whereas MIP-1α was also expressed by a high percentage of infiltrating cells. RANTES (Regulated upon activation, normal T cell expressed and secreted) and IL-8 are two other chemokines that were also detected in the salivary tissues, although both were expressed by only a low percentage of infiltrating cells (145). The chemokines expressed by the cells of the inflammatory infiltrates may attract additional leukocytes to the site of inflammation, which may contribute to the perpetuation of sialoadenitis, as will be discussed hereafter.

Table 3. Cytokine and chemokine expression in salivary glands of patients with Sjögren's syndrome

Cytokines expressed			Technique	Remarks	References
Total gland	Focal infiltrate	Epithelium			
IL-2, IFN-γ, IL-10, IL-6, TGF-ß, IL-4, IL-5, IL-12	MSG T cell clones: IL-2, IFN-γ, IL-10 (IL-4, IL-5)		RT-PCR	IL-4, IL-5, and IL-12 not in all MSG	(141)
IL-2, IFN-γ, IL-10, TNF-α, TGF-ß, IL-18, IL-12			RT-PCR	IL-4 totally absent, IL-10 and TGF-ß abundant	(143)
IL-10, IL-13			RT-PCR	Only these Cytokines examined	(142)
	MSG $CD4^+$ T cell clones: IL-2, IFN-γ, IL-10		ELISA		(140)
	IL-1α, IL-1ß, TGF-ß, GM-CSF	IL-1α, IL-1ß, IL-8, TGF-ß, GM-CSF (acini < ducts)	IHC	Similar picture in chronic sialoadenitis (CS)	(199)
	IL-2, IFN-γ, IL-10 little IL-4 and IL-5	IL-1α, TNF-α, IL-6	RT-PCR	Major salivary glands	(138)
	IL-1ß, TNF-α, IL-2, IL-6, IFN-γ, TGF-ß	IL-1ß, IL-6	ISH, IHC (IL-2 and IL-4)	Also expression of IL-2R, no IL-10 detected	(139)
	IL-2, IFN-γ, IL-10, IL-6, TNF-α, TGF-ß1	IL-2, IFN-γ, IL-10, IL-6, TNF-α, TGF-ß1	Microdissection, RT-PCR/ Southern blot	Except for IFN-γ, same cytokines also found in controls	(245)
IL-1α, IL-1ß, IL-2, IL-6, IL-8, IL-10, IFN-γ, TNF-α			RT-PCR	No IL-4, IL-13, IL-1α, IL-1ß, IFN-γ not in CS	(246)
	IL-1ß, TNF-α, IL-6	IL-1ß, TNF-α, IL-6, IFN-γ	IHC	No detection of IL-1α, IL-4, TNF-ß	(176)
IL-2, IL-6, IL-10, TGF-ß IL-6, IL-10, TGF-ß			RT-PCR ELISA	TGF-ß also in controls. Decreased in heavily infiltrated glands	(144)
	MIP-1α, MIP-1ß, IL-8, RANTES	MIP-1α, MIP-1ß, RANTES	IHC	Expression predominantly by ducts and infiltrates	(145)

RT-PCR: Reverse transcriptase-polymerase chain reaction; IHC: Immunohistochemistry; ISH: *In situ* hybridization; ELISA: Enzyme-linked immunosorbent assay.

Pathogenesis

Factors that may play a role in the pathogenesis of Sjögren's syndrome have been studied intensively. These include exogenous factors, like viruses, as well as endogenous elements that may predispose to the development of autoimmune disorders. Endogenous factors contributing to the development of autoimmune diseases may reside in the immune system itself. Recognition of self, followed by activation of autoreactive lymphocytes and initiation of an autoimmune response - as opposed to induction of tolerance - may result from a deviation in the T cell receptor repertoire or from defective expression of pro- or anti-inflammatory cytokines by leukocytes. In addition, alterations intrinsic to the APC, such as increased expression of costimulatory molecules or altered antigen processing, may lower the threshold for the induction of an autoimmune response. However, the idea that the development of autoimmune diseases is solely due to defects in the immune system is deeply ingrained and represents a strong bias. Evidence is now accumulating suggesting an important role for the target organ of the autoimmune disease in the initiation of the autoimmune reaction. For example, in two animal models of autoimmune diabetes, the BioBreeding (BB) rat and the nonobese diabetic (NOD) mouse, pancreatic ß-cell hyperreactivity and development of hyperplastic islets appear to precede islet infiltration by leukocytes (146-148). Furthermore, preferential early accumulation of APC and lymphocytes around the hyperplastic islets was demonstrated in NOD mice (149). The accumulation of APC and lymphocytes may be followed by initiation of the autoimmune response, ultimately leading to ß-cell death and the development of diabetes. The target organs in Sjögren's syndrome, the salivary and lacrimal glands, may in a similar way contribute to the development of sialoadenitis and dacryoadenitis, probably due to additional defects in the immune system. Indeed, in the NOD mouse model for Sjögren's syndrome, an important role has been suggested for genetically programmed abnormalities in the submandibular gland (150, 151).

Factors, implicated in the pathogenesis of Sjögren's syndrome will now be discussed. Attention will be paid to factors that may play a role in the initiation of the autoimmune response, but also to mechanisms that are thought to contribute to a decreased secretory response in the late phase of the autoimmune disease. Knowledge of events that induce loss of secretory function is essential for the development of new drugs, aimed at alleviation of the patients symptoms.

Viruses

Several viruses have been implicated in the pathogenesis of Sjögren's syndrome. Epstein-Barr virus (EBV) associated antigens have been found in epithelial cells of salivary gland biopsies of patients with Sjögren's syndrome, whereas they were absent in salivary tissue from controls (152, 153). By *in situ* hybridization and immunohistochemistry, EBV DNA and an EBV protein were detected in salivary gland samples of 4 of 7 patients with Sjögren's syndrome, whereas no positive signal was found in controls (154). Others reported the presence of EBV and/or human cytomegalovirus (HCMV) DNA in MSG of almost 50% of patients with Sjögren's syndrome or non-specific sialoadenitis (155). However, no difference

between the two patient groups was observed, and the authors therefore concluded that a role for these viruses in the etiology of Sjögren's syndrome is not likely. To hepatitis C virus (HCV), antibodies have been described in patients with Sjögren's syndrome. The prevalence varied between 14 and 19% (156). When salivary glands of patients with chronic HCV-induced liver disease were examined, focal lymphocytic sialoadenitis was found in 57% of the patients, and only in 5% of controls, demonstrating that sialoadenitis appears to be common in this group of HCV infected patients (157). The involvement of an unknown retrovirus similar to human immunodeficiency virus (HIV) in the pathogenesis of Sjögren's syndrome was suggested following the observation that labial salivary glands of 7 of 15 patients contained an epithelial cytoplasmic protein, reactive with a monoclonal antibody to an HIV associated protein. HIV genes were not detected in salivary glands of these patients (158). In a group of 74 Japanese Sjögren's patients from an area heavily endemic for human T-lymphotropic virus-1 (HTLV-1), 17 patients (23%) were HTLV-1 seropositive. This was significantly higher when compared with a group of blood donors (3%), whereas this control group did not differ significantly from patients with SLE. The authors suggested that HTLV-1 may be involved in the pathogenesis of Sjögren's syndrome in Japanese patients living in this endemic area (159).

Despite many studies that have been initiated on this subject, no consistent picture does emerge, and evidence for a direct pathogenic role of viruses in Sjögren's syndrome remains to be demonstrated. Still, a contribution of viruses can be envisaged. Viral infection of salivary gland epithelial cells may lead to production of IFN-γ by virus-specific T lymphocytes. Exposure of salivary epithelium to IFN-γ can have a number of effects. First, IFN-γ has been shown to induce or upregulate HLA-DR expression in a human salivary gland (HSG) epithelial cell line and in salivary gland derived primary cell cultures (160-162). Aberrant epithelial HLA-DR expression has been demonstrated in MSG of patients with Sjögren's syndrome (130, 161, 163). Salivary gland epithelial cells expressing HLA-DR molecules may function as so called non-professional APC that can present autoantigens and (re)activate autoreactive lymphocytes. Second, exposure of HSG cells to IFN-γ in the presence or absence of TNF-α has been shown to lead to a reduction in cell numbers *in vitro*, resulting from increased cell death by apoptosis as well as by necrosis (164). Increased cell death could provide a source of extracellular nuclear antigens, such as SS-A/Ro and SS-B/La, and in this way contribute to the pathogenesis of the disease. Third, incubation of HSG cells with IFN-γ resulted in the induction of protein and mRNA expression of the proinflammatory cytokines IL-1ß, IL-6 and TNF-α (160). Local production of IL-1ß and TNF-α may lead to tissue injury, as well as to increased vascular permeability and activation of leukocytes, which may result in perpetuation of the autoimmune reaction.

An additional mechanism via which viral infection could play a role in the pathogenesis of Sjögren's syndrome was postulated following observations that viral infection of cells affects the localization of the SS-B/La protein, which is normally predominantly expressed in the nucleus. Infection of the monkey kidney derived CV-1 cell line with herpes simplex virus type I resulted in translocation of SS-B/La to the cell surface (165). In the hepatic Hep-2 cell

line, accumulation of SS-B/La at the periphery of the nucleus was observed 24 hours after infection with adenovirus 2, whereas cytoplasmic and membranous localization was observed 48 hours after infection (95). Salivary gland epithelial cells infected with adenovirus demonstrated an altered nuclear staining of SS-B/La when compared with noninfected cells, whereas cytoplasmic localization was observed following incubation with IFN-γ (166). Primary salivary gland cultures exposed to IFN-γ also revealed increased cytoplasmic localization of SS-B/La (161). The appearance of SS-B/La on the surface of virally infected epithelial cells together with IFN-γ induced expression of HLA-DR could form the basis of a T cell dependent mechanism for anti-SS-B/La autoantibody production.

Genetic factors - HLA association

Evidence for a genetic factor that may contribute to the development of Sjögren's syndrome stems from studies describing families in which 2 or more members are affected by Sjögren's syndrome (167-169). Studies on the role of genetics in Sjögren's syndrome have mainly focussed on the association between different HLA genes and Sjögren's syndrome. It has been shown that in a group of Caucasian patients with Sjögren's syndrome, HLA-DR3 and HLA-DR4 alleles were increased in different subgroups of patients, whereas an increased frequency of HLA-DR3 and HLA-DR2 has been described in relatives of patients with Sjögren's syndrome (167, 168, 170). A large study among patients in California also revealed an increased frequency of HLA-DR3 (in addition to certain HLA-DQ alleles) in patients with primary Sjögren's syndrome. Furthermore, these alleles were shown to be associated with anti-SS-A/Ro and anti-SS-B/La antibodies, and with clinical and laboratory findings (171). A strong association between particular HLA-DQ and HLA-DR alleles and the presence of anti-SS-A/Ro and anti-SS-B/La has also been demonstrated by others. In these studies, no differences were found between patients with Sjögren's syndrome and SLE, and evidence was presented suggesting that distinct HLA class II alleles may influence diversification of the autoimmune response to the La/Ro RNP (172-174). When groups of Sjögren's patients of different ethnic backgrounds were examined, increased frequencies of specific HLA-DR and HLA-DQ alleles were found in each ethnic group, but no single allele was increased in all patient groups. Although a unique HLA class II allele probably was not required for the development of Sjögren's syndrome, it was suggested that the disease could arise as a consequence of exposure to an environmental agent in a patient with an allele, common in that ethnic population (175). In conclusion, although increased frequencies of particular HLA gene products in groups of patients with Sjögren's syndrome have been described, no consensus exists on a unique HLA gene in Sjögren's syndrome.

Cytokines/ chemokines

Immune mediators such as cytokines and chemokines play an essential role in the maintenance of immunological homeostasis whereas dysregulated expression may contribute to the pathogenesis of immune disorders, such autoimmune diseases and allergies. Altered expression of cytokines in the tissue before infiltration with lymphocytes could play a role in

the initiation of the autoimmune response. In addition, following the onset of disease, cytokines released from infiltrating cells in inflamed tissues may induce destructive changes in the tissues or attract additional leukocytes. As mentioned before, infiltrating lymphocytes in MSG of patients with Sjögren's syndrome were found to express the Th1 cytokines IL-2 and IFN-γ, in addition to the Th2 cytokines IL-6 and IL-10 (138-140, 176). Cytokines that were most consistently expressed by the salivary gland epithelial cells were the proinflammatory cytokines IL-1α, IL-1ß, IL-6, and TNF-α (138, 139, 176). These cytokines were also detected in saliva of patients with Sjögren's syndrome (138).

A role for cytokines expressed by infiltrating lymphocytes as well as by glandular epithelial cells in the pathogenesis of Sjögren's syndrome can be envisaged. Production of IL-2 by activated Th1 lymphocytes can induce proliferation of additional T lymphocytes, whereas IFN-γ can have a number of effects. These include increased HLA class II expression on salivary gland epithelial cells as well as on APC, induction of cell death, and of proinflammatory cytokine expression. Furthermore, IFN-γ can activate macrophages, resulting in increased expression and release of inflammatory mediators by these cells, such as IL-1 and TNF-α. These cytokines can induce tissue damage, but can also stimulate the expression of other cytokines, among which IL-6. The production of IL-6 and IL-10 by activated Th2 cells can stimulate B cells to proliferate and differentiate, ultimately resulting in (auto)antibody production, whereas IL-6 can also upregulate IL-2R expression by T lymphocytes, leading to increased responsiveness of T cells to IL-2.

The proinflammatory cytokines IL-1, IL-6, and TNF-α can mediate a variety of effects in the salivary glands. First, local inflammatory effects of the cytokines IL-1 (α and ß) and TNF-α include induction of adhesion molecule expression on endothelial cells, and vasodilatation, resulting in extravasation of leukocytes from the vascular compartment, and migration to the site of inflammation due to expression of chemoattractants. This mechanism has been proposed to contribute to the homing of autoreactive $CD4^+$ lymphocytes to the salivary glands of patients with Sjögren's syndrome (138). Second, expression of IL-1ß and IL-6 by glandular epithelial cells , in which increased HLA-DR expression can be induced by IFN-γ (130, 161, 163), theoretically enables these cells to act as APC, a function that has also been proposed for salivary gland epithelial cells and will be discussed hereafter (177).

Third, *in vitro*, both IL-1ß and TNFα have been shown to induce increased protein and mRNA expression of matrix metalloproteinase (MMP)-2 by salivary gland epithelial cells, whereas expression of tissue inhibitor of metalloproteinase (TIMP)-2 was decreased (178). Increased levels of MMP-9, the expression of which can also be influenced by cytokines, have been measured in saliva and in labial salivary glands of patients with Sjögren's syndrome as compared with controls (179, 180). An altered balance between MMP and their inhibitors could lead to degradation of the basement membrane, and thereby to disturbed proliferation or apoptosis of the epithelial glands. Fourth, the cytokines TNF-α, IL-1, and IFN-γ have been demonstrated to induce expression of inducible NOS (iNOS) in hepatocytes and macrophages (181, 182). Increased iNOS activity will stimulate NO production that may induce damage to epithelial cells of the exocrine cells. Indeed, increased nitrite levels, indi-

cating increased NO production, have been described in saliva of patients with Sjögren's syndrome as compared with controls, suggesting that iNOS expression is induced in the salivary gland of patients with Sjögren's syndrome (183).

Another pathway, by which cytokines may cause damage to salivary gland epithelial cells is via the induction of Fas expression on the epithelium. This can be followed by ligation by Fas ligand, present on activated T lymphocytes, and induction of apoptosis in the Fas expressing cell. Cytokine mediated upregulation of Fas expression on thyrocytes, followed by Fas/FasL interaction and induction of apoptosis has been suggested to be responsible for tissue damage and clinical hypothyroidism in Hashimoto's thyroiditis. IL-1ß was identified as the most important cytokine in this process (184, 185). Enhanced Fas expression on the colon carcinoma epithelial cell line HT-29 could be induced by IFN-γ and TNF-α (186), whereas IL-1ß, IFN-γ, or a combination of IL-1ß, IFN-γ and TNF-α could upregulate Fas expression in NOD pancreatic islet cells (187). Exposure of HSG cells to IFN-γ and/or TNF-α resulted in increased Fas expression and susceptibility to anti-Fas mediated cell death (188, 189). Furthermore, TNF-α has been shown to induce apoptosis in HSG cells, which occurred along with decreased expression of X chromosome-linked inhibitor of apoptosis protein (XIAP) in this cell line (190).

Infiltrating mononuclear cells as well as ductal epithelial cells were identified as important sources of the chemokines MIP-1α, MIP-1ß and RANTES in MSG of patients with Sjögren's syndrome, but not of healthy controls (145). Although the role of these chemokines in the pathogenesis of Sjögren's syndrome remains to be elucidated, chemokines can orchestrate leukocyte recruitment by regulation of adhesion molecule expression on vascular endothelium, transendothelial migration and chemotactic movement to the site of inflammation, thereby contributing to perpetuation or exacerbation of the local autoimmune reaction (191). Leukocyte subsets that will mainly accumulate due to expression of MIP-1α, MIP-1ß and RANTES in the MSG are monocytes/macrophages and T lymphocytes, which are preferentially attracted by ß-chemokines, the subfamily to which these chemokines belong. Whereas MIP-1α, MIP-1ß and RANTES are inflammatory cytokines, mainly expressed in response to a particular pathogen or damage, lymphoid chemokines are involved in the homeostatic trafficking of leukocytes into different lymphoid compartments. The expression of the latter group of chemokines in MSG has been studied as well (192). Lymphoid chemokines that were specifically expressed in MSG of patients with Sjögren's syndrome include secondary lymphoid organ chemokine (SLC), B cell attracting chemokine (BCA)-1, Epstein-Barr virus-induced gene 1 ligand chemokine (ELC), and pulmonary activation regulated chemokine (PARC). The main sources of these chemokines were ductal epithelial cells and infiltrating mononuclear cells. The authors suggested that these chemokines may play a role in the organization of lymphoid structures in Sjögren's syndrome (192).

Adhesion molecules

Circulation and accumulation of leukocytes, as well as cell-cell interactions are mediated by cell adhesion molecules (193-195). In autoimmune diseases, the expression of adhe-

sion molecules on leukocytes and endothelial cells may play an essential role in the homing of leukocytes to the target organ, and perhaps influence the cellular composition of the inflammatory infiltrates. Additionally, adhesion molecule expression on glandular epithelium may result in increased interactions between leukocytes and epithelial cells, and in perpetuation of the autoimmune response. For this reason, the expression of adhesion molecules in exocrine glands of patients with Sjögren's syndrome has been the subject of a number of studies.

Several studies reported the expression of intercellular adhesion molecule (ICAM)-1 on an increased percentage of epithelial and endothelial cells in MSG of patients with Sjögren's syndrome as compared with controls, although expression was weak. Furthermore, the majority of infiltrating mononuclear cells expressed significant levels of ICAM-1 and its ligand lymphocyte function associated antigen (LFA)-1 (196-199).

Messenger RNA expression of vascular cell adhesion molecule (VCAM)-1 was exclusively found in salivary and lacrimal gland biopsies of patients with Sjögren's syndrome, but not of controls (197). Immunohistochemistry revealed expression on endothelial structures and mononuclear cells only. Very late antigen (VLA)-4, the ligand for VCAM-1, was expressed on the majority of infiltrating $CD4^+$ lymphocytes (197, 198). Increased expression of ICAM-1 and VCAM-1 in MSG of patients with Sjögren's syndrome was suggested to be the consequence of expression of inflammatory cytokines in the glands, since overexpression of these molecules was observed concurrently with IFN-γ and IL-1ß expression in the glands (197). Indeed, upregulation of ICAM-1 expression on cultured salivary gland epithelial cells upon exposure to IFN-γ has been observed (196).

CD2 expression has been demonstrated on infiltrating mononuclear cells in MSG of patients with Sjögren's syndrome, whereas the ligand LFA-3 was detected on infiltrating cells and a large number of acinar and ductal epithelial cells (196, 198). It was suggested that LFA-3 could play an important role in binding of $CD2^+$ lymphocytes and consequent T cell activation (198). Expression of both CD2 and LFA-3 was also detected in MSG of patients with chronic sialoadenitis. No difference was found between chronic sialoadenitis and Sjögren's patients (199).

In conclusion, these studies demonstrate changes in adhesion molecule expression in glandular biopsies of patients with Sjögren's syndrome. The adhesion molecules VCAM-1 and ICAM-1, expressed by endothelial structures, may contribute to the recruitment of VLA-4^+ and LFA-1^+ lymphocytes and be the main targets for T cell migration to the exocrine tissues in Sjögren's syndrome. Increased expression of adhesion molecules may be mediated by proinflammatory cytokines expressed in the inflamed salivary glands, and occur secondarily to the development of lymphocytic infiltrates, although a role in the initiation of sialoadenitis can not be ruled out.

Apoptosis

Two major pathways have been described leading to the induction of apoptosis in cells. The so-called extrinsic apoptosis pathway involves ligation of receptors in the cell

membrane, such as Fas or other members of the TNF receptor family by their corresponding ligands. Ligation of these receptors leads to trimerization and aggregation of FADD (Fas-associated protein with death domain) and procaspase-8 to the receptor complex. Binding of procaspase-8 results in activation of the protease domain, which will subsequently activate effector proteases such as caspase-3. These effector caspases can cleave vital cellular substrates, ultimately resulting in apoptosis (Fig. 1A) (reviewed in 200). Pro-apoptotic members of the bcl-2 protein family, such as bax, can induce apoptosis via the intrinsic pathway, in which these proteins target to the mitochondria and induce the release of cytochrome c into the cytosol (Fig. 1B) (201, 202). Cytochrome c binds to the caspase-activating protein Apaf-1 (apoptotic protease-activating factor), which in turn activates caspase-9. Activated caspase-9 can subsequently cleave and activate the effector molecule caspase-3, which can mediate substrate cleavage (Fig. 1B) (202-204). Although originally described as two separate apoptosis pathways that function independently of each other, evidence has now accumulated that suggests interactions between components of the two pathways. Fas ligation has been described to result in the formation of pores in the mitochondrial membrane and in the subsequent release of cytochrome c and other apoptosis factors in the cytosol (205). Using a human breast epithelial cell line, others demonstrated that translocation of the proapoptotic molecule bax from the cytosol to the mictochondrial membrane occurs as part of the Fas-induced apoptotic pathway. Insertion of bax into the mitochondrial membrane could be inhibited by bcl-2 overexpression (206). In addition to the extrinsic and intrinsic apoptosis pathways, apoptosis can be induced in cells via the release of perforin and granzyme containing granules. This pathway is responsible for a major part of the cytotoxicity generated by $CD8^+$ T lymphocytes (reviewed in 207). In the presence of calcium, perforin polymerizes and forms channels in the cell membrane, through which granzymes may pass. These granzymes can subsequently activate the death machinery of target cells. Granzyme B for example has been demonstrated to efficiently activate effector caspases (reviewed in 207, 208), but also to be able to directly cleave nuclear substrates of caspases and induce apoptosis in a caspase-independent way (209).

Although apoptosis is a physiological process, amongst others involved in maintenance of homeostasis, disturbances in this process can play a significant role in the pathogenesis of autoimmune diseases. First, defective expression of Fas and FasL in the *lpr* and *gld* mouse strains, respectively, results in severe systemic autoimmune manifestations (210). Second, apoptosis may participate in the initiation of an autoimmune response by the release of nuclear antigens, or via the generation of neoantigens by activated caspases. Third, apoptosis may be an important mechanism inducing cell death in the late phase of an autoimmune response. Fourth, abnormalities in apoptosis may contribute to the chronic nature of autoimmune processes. Increased expression of anti-apoptotic factors such as bcl-2 by infiltrating lymphocytes can protect these cells to apoptosis, resulting in the persistence of the infiltrates and continuation of the autoimmune process.

The potential contribution of apoptosis to the induction of the autoimmune reaction is receiving more and more attention. In the past, apoptosis was mainly regarded as a way of

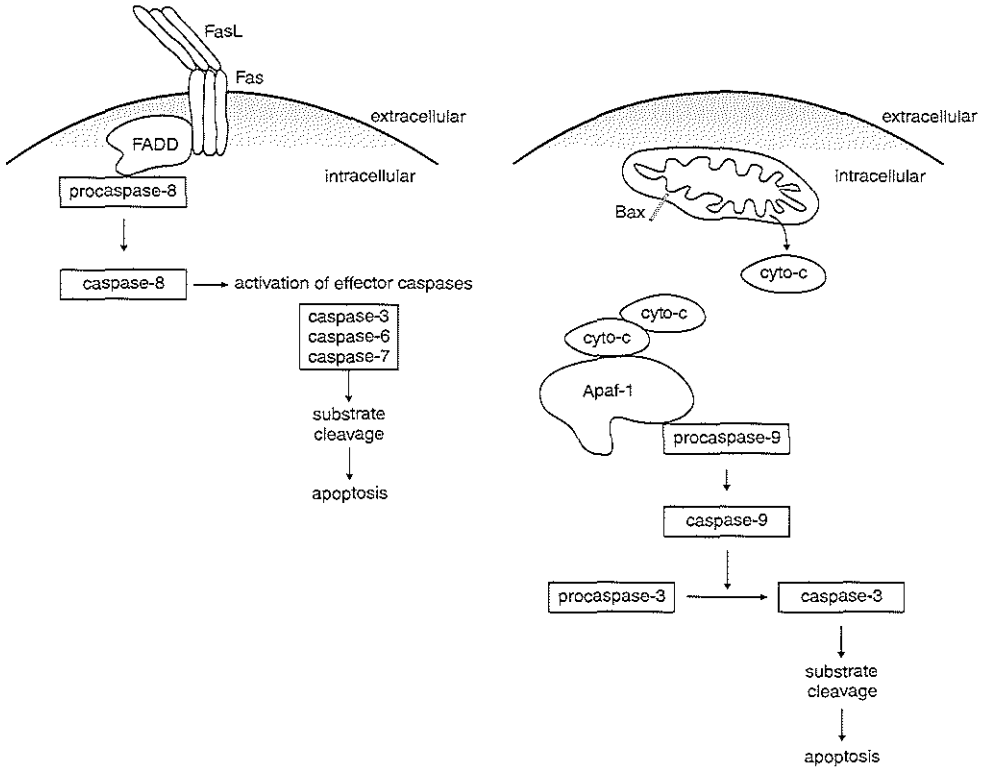

Figure 1
Two major pathways that can lead to the induction of apoptosis. Apaf-1: apoptotic protease-activating factor; Cyto-c: cytochrome c; FADD: Fas-associated protein with death domain; FasL: Fas ligand.

cell death not inducing an inflammatory response. However, several lines of evidence have now demonstrated that an immune response can evolve following the induction of apoptosis (211, 212). First, apoptosis can induce the exposure of antigens, normally retained within the cytoplasm or nucleus of the cell. These antigens become accessible for autoreactive lymphocytes, possibly resulting in the initiation of an autoimmune response. Evidence for this possibility was obtained in experiments in which mice were injected intravenously with syngeneic apoptotic thymocytes. These mice developed an autoantibody response, including low levels of antinuclear antibodies and anti-ss-DNA antibodies (212). Furthermore, surface blebs of apoptotic keratinocytes and cardiocytes were found to contain SS-A/Ro and SS-B/La, which are normally present in the nucleus or cytoplasm of the cell (96, 99). This suggests that in patients with Sjögren's syndrome or SLE, systemic exposure to (increased numbers of) apoptotic cells could result in the development of autoantibodies to these generalized antigens. A second pathway by which apoptosis may contribute to the induction of an autoim-

mune response is via the activation of caspases. These enzymes can cleave intracellular proteins, which may result in the exposure of cryptic antigens and in the subsequent induction of an autoimmune reaction. A protein that could be a substrate for apoptosis associated caspases is 240 kD α-fodrin, a normal component of the cytoskeleton. It has been demonstrated that this protein was cleaved following induction of apoptosis in a T cell hybridoma, resulting in the generation of 120 kD sized α-fodrin (105, 106). As mentioned before, antibodies to 120 kD α-fodrin have been described in the serum of a high percentage of patients with Sjögren's syndrome (103, 107). The direct involvement of the 120 kD form of the protein in the pathogenesis of Sjögren's syndrome was suggested following the identification of 120 kD α-fodrin in MSG of patients with Sjögren's syndrome, whereas it was absent in control tissues. In addition, immunization of neonatal NFS/sld mice thymectomized 3 days after birth, with a recombinant α-fodrin peptide, inhibited the development of sialoadenitis normally observed in this mouse model (103). Although these experiments are indicative for a role of 120 kD α-fodrin in the initiation of the autoimmune response, the direct pathogenic role in Sjögren's syndrome in humans still remains to be demonstrated. However, the fact that induction of apoptosis leads to the processing of a protein that may be involved in the pathogenesis of Sjögren's syndrome illustrates the potential contribution of enzymes, activated in association with apoptosis, to the generation of cryptic antigens.

The involvement of apoptosis in the induction of glandular damage in the late phase of the autoimmune disease has clearly been demonstrated in patients with Hashimoto's thyroiditis, in which thyrocytes that constitutively express FasL can induce apoptosis in neighbouring thyrocytes in which Fas expression was induced by IL-1ß (184). The infiltrating T lymphocytes were not directly involved in thyrocyte destruction, but were subject to Fas-mediated apoptosis themselves (213). Other autoimmune diseases in which apoptosis has been suggested to contribute to damage to the target organ include insulin-dependent diabetes mellitus (IDDM) and multiple sclerosis (MS) (214, 215). In contrast to Hashimoto's thyroiditis, the cells responsible for the induction of apoptosis in patients with IDDM were activated T lymphocytes. Apoptotic ß-cells in pancreata of these patients were in close proximity of FasL positive T lymphocytes, whereas endocrine and exocrine cells were FasL negative (214).

In the majority of studies on the role of apoptosis in the pathogenesis of Sjögren's syndrome, apoptotic epithelial cells (both ductal and acinar) were detected in MSG of patients, whereas apoptosis was decreased or even absent in epithelial cells of control biopsies (216-219). In addition, apoptotic cells were identified among infiltrating mononuclear cells in salivary glands of Sjögren's patients, although the number was generally low (216, 217, 219-222). Expression of Fas and FasL was observed on acinar and ductal epithelial cells as well as on infiltrating lymphocytes. Fas expression on epithelial cells was increased when compared with controls, whereas expression of FasL was not detected in biopsies in which no lymphocytic infiltration had occurred (188, 216, 217, 219). It was suggested that abnormal epithelial coexpression of Fas and FasL in Sjögren's syndrome could result in the induction of apoptosis in epithelial cells, mediated by adjacent epithelial cells, similar to the situation

in Hashimoto's thyroiditis. Alternatively or in addition, FasL expressed on activated lymphocytes could also mediate Fas ligation and activate the death pathway in the epithelial cells (Fig. 2A). Low numbers of apoptotic cells in the lymphocytic infiltrates despite relatively high expression of Fas and FasL may be due to overexpression of the anti-apoptotic factor bcl-2 on these lymphocytes. Epithelial expression of bcl-2 was low as compared with control tissue or even absent, resulting in increased susceptibility to apoptosis inducing signals (Fig. 2A) (190, 216, 218, 220, 221).

Another pro-apoptotic factor that has been implicated in Sjögren's syndrome is bax. Expression of this protein was increased in infiltrating mononuclear cells and epithelial cells (220, 221). The pro-apoptotic effect of bax in infiltrating cells could be overcome by co-expression of bcl-2, since infiltrating bax positive mononuclear cells expressing elevated levels of bcl-2 were not apoptotic, whereas weak bcl-2 expression was not sufficient to inhibit the pro-apoptotic effect (Fig. 2B) (221). The anti-apoptotic factor X chromosome-linked inhibitor of apoptosis protein (XIAP) was recently proposed to be involved in the regulation of apoptosis in epithelial cells of patients with Sjögren's syndrome. XIAP was expressed by acinar and ductal epithelial cells in MSG of patients, whereas expression was absent in controls (190). This protein can inhibit the activity of effector caspases, such as caspase-3 and caspase-7, as well as activation of caspase-9 (Fig. 2C) (200, 223, 224). Interestingly, incubation of HSG cells with TNF-α resulted in decreased XIAP expression, and increased apoptosis, an effect that could be overcome by the cytokines IL-1ß, IL-10 and TGF-$ß_1$ (190).

Apoptosis in target cells can also be induced by the secretion of cytolytic granules containing perforin and granzyme B by activated T cells (Fig. 2D). Both cytotoxins have been demonstrated in infiltrating mononuclear cells in MSG of patients with Sjögren's syndrome, but not in glands of patients with aspecific sialoadenitis (220). In another study, 50% of acinar epithelial cells positive for an early apoptotic marker, were in contact with $CD8^+$ T lymphocytes, which suggests that these cells play an important role in the induction of apoptosis. The $CD8^+$ T cells were strongly positive for the integrin adhesion molecule $\alpha_E ß_7$, which could play a role in the adhesion of $CD8^+$ T cells to E-cadherin on target cells. Around acinar cells being in close contact with the $\alpha_E ß_7{}^+CD8^+$ T lymphocytes, perforin and granzyme B were expressed, also indicating that this pathway is likely to be involved in the induction of damage to acinar cells by cell-cell contact (225). NO is a mediator, that when produced in excessive amounts, can induce apoptosis in a variety of cell types (226-228). In the joint of patients with rheumatoid arthritis, a close correlation between the presence of iNOS and apoptosis in the synovial lining layer has been demonstrated, and apoptosis could be decreased in culture explants of cartilage and synovium by the iNOS inhibitor L-N^G-monomethylarginine (L-NMMA) (229). Although a direct association between NO production and apoptosis in Sjögren's syndrome has not been examined, NO is produced in salivary glands of patients with Sjögren's syndrome as evidenced by increased nitrite levels in the saliva of patients. A role for this mediator in the induction of apoptosis in diseased glands can be envisaged (183). Macrophages and salivary gland epithelial cells have been suggested to be important sources of NO production in the salivary gland, and cytokines were postulated to

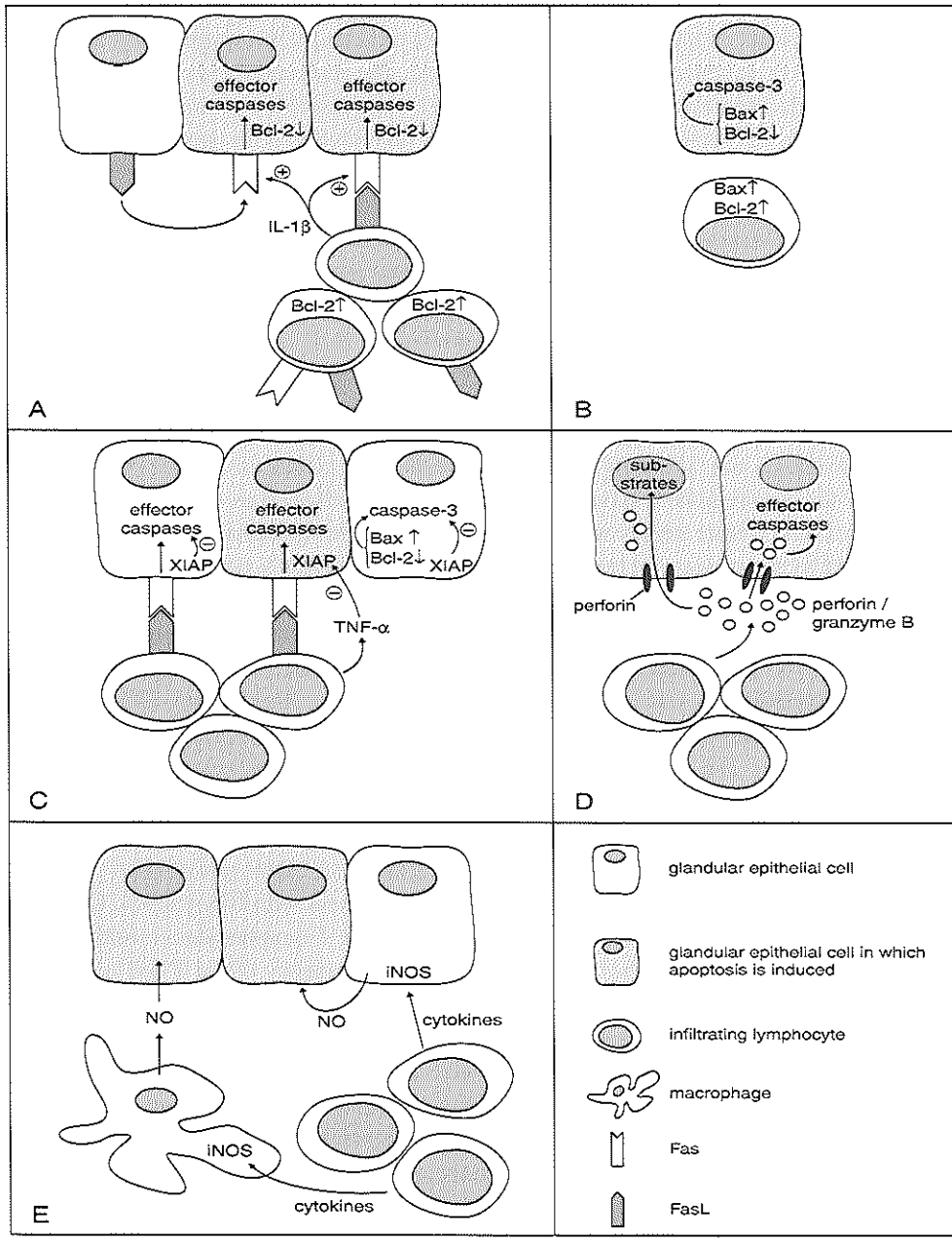

Figure 2
Cells and molecules proposed to be involved in the regulation of apoptosis in Sjögren's syndrome. FasL: Fas ligand; IL-1ß: interleukin-1ß; iNOS: inducible nitric oxide synthase; NO: nitric oxide; XIAP: X chromosome-linked inhibitor of apoptosis protein.

contribute to NO production, via the induction of iNOS expression (Fig. 2E) (183).

In conclusion, apoptosis may be an important mechanism responsible for cell death in exocrine glands of patients with Sjögren's syndrome. The molecules and cells, postulated to be involved in the regulation of apoptosis in Sjögren's syndrome are summarized in figure 2. However, despite the number of studies in which an important role for apoptosis in the effector phase of Sjögren's syndrome is implied, controversy among this subject remains. The range between reported apoptotic frequencies is high (0.3% to 68%) (216, 218-220, 222, 230), and apoptosis has even been described to be a rare event in Sjögren's syndrome, despite expression of Fas and FasL on epithelial and infiltrating mononuclear cells (231). This indicates that further investigations on the role of apoptosis are needed before definite conclusions on its role in Sjögren's syndrome can be drawn.

Epithelial cells as antigen presenting cells

Diagnosis of Sjögren's syndrome is dependent on symptoms and/or signs of oral and ocular involvement, but, although the salivary and lacrimal glands are the organs most often affected by the autoimmune response, other organs can be affected as well. The observation that involvement of other organs was mainly restricted to the epithelial cells of these organs was followed by the suggestion that the affected tissue in Sjögren's syndrome is the epithelium. Therefore, a more descriptive pathophysiological term 'autoimmune epithelitis' was proposed (177). An active role for the epithelium in the initiation and perpetuation of the autoimmune reaction was postulated following the demonstration of HLA-DR and proinflammatory cytokine expression by salivary gland epithelial cells (130, 138, 139, 161, 232). When the expression of costimulatory molecules was examined in MSG biopsies of patients with Sjögren's syndrome and compared with biopsies of patients with aspecific sialoadenitis, expression of CD80 and CD86 was evident on an increased percentage of acinar and ductal epithelial cells (in addition to mononuclear cells) in Sjögren's patients. In long term epithelial cell cultures obtained from MSG, expression of CD80 and CD86, and of HLA-A, -B, -C, and HLA-DR was upregulated or induced following treatment with IFN-γ (162). CD80 and CD86 can interact with CD28 and CTLA-4, expressed on T lymphocytes, resulting in the delivery of a second signal to the T cell (in addition to occupancy of the T cell receptor by peptide-HLA complexes). Since costimulatory signals are required during the interaction of an APC with a T lymphocyte in order to induce T cell activation (as opposed to induction of T cell anergy), salivary gland epithelial cells were proposed to behave as non-professional APC (162). Although naïve T lymphocytes require strong costimulatory signals for their activation that can only be delivered by professional APC, epithelial cells might function as APC once lymphocytic infiltrates have developed in the salivary glands. The production of IFN-γ by infiltrating T cells may induce increased epithelial expression of HLA-DR and costimulatory molecules. The concurrent induction of cell death in adjacent epithelial cells, possibly also mediated via IFN-γ (164), might result in the presentation of salivary gland derived antigens to T cells that have already encountered antigen, leading to perpetuation of the autoimmune reaction.

Sicca symptoms, result of functional quiescence as opposed to glandular damage?

The mechanism(s) underlying diminished function of exocrine organs in Sjögren's syndrome is unknown, but damage invoked to the glandular epithelial cells by the focal infiltrates was commonly thought to be responsible for sicca symptoms. Death of the epithelial cells could result from the release of cytotoxic mediators or from apoptosis, induced by infiltrating cells, as discussed in the previous section (216-220, 225). However, several lines of evidence argue against this traditional view. First, although significant numbers of apoptotic cells have been demonstrated in MSG of patients with Sjögren's syndrome (216-219), a negative correlation between the degree of glandular damage and the secretory response has never been observed. Second, no correlation was found between the degree of lymphocytic infiltration in MSG and salivary flow (233, 234). Third, salivary glands have a substantial functional reserve, and some degree of atrophy can be tolerated without a reduction in salivary flow rate. This is also illustrated by the fact that in the majority of mouse models for Sjögren's syndrome, a decreased secretory response does not develop, despite extensive lymphocytic infiltration in the salivary glands (see chapter on mouse models for Sjögren's syndrome). Thus, destructive changes in the exocrine glands are unlikely to fully account for sicca symptoms.

Alternatively, sicca symptoms may be the result of a diminished capacity of salivary gland acinar epithelial cells to respond to stimuli, or of a decrease in agonistic stimuli reaching the epithelial cells. Cholinergic dysfunction, which may contribute to sicca symptoms, has been demonstrated in a high percentage of patients with Sjögren's syndrome. The microvascular response to the muscarinic cholinergic agonist carbachol, administered to the skin of the forearm of Sjögren's patients, was significantly decreased when compared with controls (235). At the level of the exocrine glands, cholinergic dysfunction was proposed to be induced by cytokines released by infiltrating cells. Cytokines may interfere with neural signals delivered to the epithelial cells, resulting in an altered functional response of glandular epithelial cells to neural stimulation, and impairment in the secretion of anions (236). However, evidence against a major antisecretory effect of cytokines on acinar epithelial cells in vitro has been presented (237). When conditioned medium, prepared from splenic lymphocytes exposed to concanavalin A was added to murine submandibular acinar epithelial cells, a decreased acetylcholine-evoked Ca^{2+} mobilization was found. This effect was only found following acute exposure of acinar cells to the medium, whereas chronic exposure did not influence the response. Although conditioned medium is a rich source of cytokines, this acute effect was mediated by cholinesterase, since it could be blocked by a cholinesterase inhibitor. Furthermore, the response induced by the cholinesterase-resistant muscarinic agonist carbachol was not influenced by the conditioned medium. It was suggested that *in vivo*, cholinesterase could leak from the serum into the interstitium of the salivary gland as a component of the inflammatory exudate, or be derived from the surface membrane of activated lymphocytes (237). The cholinesterase concentration in the salivary gland could rise to a point at which acetylcholine released by parasympathetic nerve terminals is metabolized

before it can bind to muscarinic receptors on the acinar cells. Prolonged loss of function, due to the action of cholinesterase, could be the trigger for glandular atrophy, which develops with progression of the disease. Atrophy would then arise as a result of diminished function, rather than being the cause of it (237).

Recently, it was demonstrated that aquaporin (AQP)-5, a member of water-specific membrane channel proteins, shows an abnormal cellular localization in MSG of patients with Sjögren's syndrome. In these glands, AQP-5, which is normally expressed on the apical membrane of acinar epithelial cells and of the proximal segment of intercalated ducts (238, 239), was primarily expressed on basal membranes of acinar cells (240). Interestingly, similar findings were done in lacrimal gland biopsies of Sjögren's patients, in which AQP-5 was mainly detected in the cytoplasm of acinar cells, in contrast to the apical distribution in controls (241). The demonstration that AQP-5 knock out mice have decreased saliva production supports the hypothesis that abnormal localization of AQP-5 may be responsible for decreased secretion of exocrine glands in patients with Sjögren's syndrome (242).

A mechanism operating at the receptorial level may involve autoantibodies directed to muscarinic cholinergic receptors that can compete with natural agonists for binding to the receptor, resulting in a diminished functional response upon agonistic stimulation. Recently, evidence has accumulated supporting an important role for an antibody mediated mechanism in the decreased secretory output observed in patients with Sjögren's syndrome. An important clue came from experiments in which injection of SjS-IgG into NOD-Igμ^{null} mice, which lack functional B lymphocytes, resulted in a decreased stimulated salivary output (117). Antibodies directed to the M3 muscarinic receptor had already been demonstrated in the serum of patients with Sjögren's syndrome (116, 119). Since the M3 muscarinic receptor is important in the stimulation of watery salivary flow, these autoantibodies could well be the humoral factor responsible for glandular dysfunction. The capability of anti-M3 muscarinic receptor antibodies to interfere with parasympathetic neurotransmission was demonstrated in experiments in which carbachol induced bladder muscle contraction could be decreased by preincubation with SjS-IgG (77). The antibodies were suggested to mediate a whole array of autonomic features reported in patients with Sjögren's syndrome, including bladder irritability, constipation, fluctuating blood pressure and dilated pupils. A major role for anti-M3 muscarinic receptor antibodies in diminished secretory function could also explain the benefit that many patients experience from treatment with the muscarinic receptor agonist pilocarpine (73-75). Although an anti-secretory effect of antagonistic anti-M3 muscarinic receptor antibodies can easily be envisaged, Bacman et al described anti-M3 receptor antibodies with agonistic properties (116, 118). These antibodies may ultimately induce desensitization or internalization of the muscarinic receptors, resulting in a decreased secretory response upon stimulation.

In addition to the defects described above, involving alterations at the receptorial or prereceptorial level that may result in diminished secretion of acinar epithelial cells in patients with Sjögren's syndrome, a postreceptorial defect has been described. When protein kinase C (PKC) isoforms were studied in MSG of patients with Sjögren's syndrome, three

PKC isoforms were defectively expressed (243). Since PKC is participating in the signal transduction pathway that is activated following ligation of muscarinic receptors (244), defective expression of PKC isoforms could influence the functional response induced by muscarinic receptor agonists.

In conclusion, although sicca symptoms in Sjögren's syndrome were traditionally thought to develop as a consequence of destruction of glandular tissue, an alternative view of exocrine dysfunction is receiving more and more attention. In this view, glandular dysfunction may arise due to either diminished capacity of the acinar epithelial cells to respond to stimuli, or to a decrease in agonistic stimuli reaching the acinar epithelial cells. This pathophysiological mechanism would greatly improve the therapeutic opportunities for the patient; after all, it assumes absence of irreversible glandular damage, and secretion may still be stimulated by chemical compounds. These may include compounds that compete with blocking antibodies, or that are insensitive to enzymes that can degrade neurotransmitters.

Concluding remarks

Sjögren's syndrome is an autoimmune disorder with a high prevalence. In addition to involvement of the salivary and lacrimal glands, other organs can be affected. Besides symptoms resulting from involvement of the exocrine glands, fatigue is a common disease manifestation which can have a major impact on quality of life. The event(s), leading to the initiation of the autoimmune response is not known, and studies on the early phase are hampered by the long time gap between initiation and diagnosis of the disease. Furthermore, the population of patients with Sjögren's syndrome is heterogeneous, and mechanisms involved in the initiation of disease may vary among patients. In the end phase of Sjögren's syndrome, a decreased production of saliva and tears may occur, which was commonly thought to be the result of destruction of glandular epithelial cells, due to the development of lymphocytic infiltrates in the glands. However, an alternative hypothesis gaining more attention, attributes an important role to anti-muscarinic receptor antibodies that can interfere with binding of natural agonistic molecules to the receptor. Since these antibodies were specifically detected in serum of patients with Sjögren's syndrome, the presence of these antibodies would not only increase therapeutic possibilities, but could also contribute to diagnosis of the disease.

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Chapter 1.2

PATHOGENESIS OF SJÖGREN'S SYNDROME: CHARACTERISTICS OF DIFFERENT MOUSE MODELS FOR AUTOIMMUNE EXOCRINOPATHY

Introduction

The pathogenesis of Sjögren's syndrome can theoretically be divided in two phases: An initiation phase, in which certain events lead to the initiation of the autoimmune reaction, and an effector phase, in which lymphocytic infiltrates develop in the salivary and/or lacrimal glands (also named sialoadenitis and dacryoadenitis, respectively) and a decreased production of saliva and tears is observed. Although a combination of immunologic, genetic, hormonal and viral factors have been implicated in the pathogenesis of Sjögren's syndrome, the events leading to the initation of the autoimmune reaction are still not known (1-3).

Because of the long time gap between the initial events leading to the activation of autoreactive lymphocytes, and the final diagnosis Sjögren's syndrome, it is virtually impossible to study the initiation phase of the autoimmune process in humans. Mouse models for Sjögren's syndrome are therefore of great value. A high percentage of the mice will develop sialoadenitis and/or dacryoadenitis, which enables investigators to study the initiation phase in detail. The salivary and lacrimal glands can be studied from birth on till the autoimmune process has fully developed. The role of molecules, thought to contribute to the initiation or effector phase of the autoimmune response can be carefully examined by administration of monoclonal antibodies to the molecule(s) of interest. Transgenic mouse models can be used to assess the influence of genetic factors on the development of disease. On the basis of these experiments, a therapy can be developed, the effectiveness of which can be evaluated in the mouse model. In a heterogeneous disease like Sjögren's syndrome, pathogenetic mechanisms underlying the development of the autoimmune disease may vary among patients. The simultaneous use of different mouse models offers the opportunity to get insight into different pathogenetic mechanisms that may underly the development of Sjögren's syndrome in subgroups of patients.

Here, we review different mouse models for Sjögren's syndrome, paying predominant attention to the NOD and the MRL/lpr mouse, with special emphasis on the submandibular glands (SMG). We discuss the pathogenetic mechanisms that may underlie the development of the autoimmune process. We believe that each mouse model exhibits unique characteristics of sialoadenitis and dacryoadenitis which can be exploited to study one particular phenomenon of Sjögren's syndrome. These characteristics may include the origin of the event(s) leading to the initiation of the autoimmune reaction, as well as the mechanism(s) responsible for perpetuation of the autoimmune process. Knowledge of strain specific characteristics of the disease will enable targeted manipulation of mechanisms that may underly the development of the autoimmune process.

Non-obese diabetic mouse

General features

The nonobese diabetic (NOD) mouse was originally bred from a subline of outbred ICR mice that were used in a breeding program of which the initial goal was the development

of a mouse strain with cataract (4). The progeny of a female mouse that spontaneously developed insulin dependent diabetes mellitus (IDDM) in association with insulitis was used as founders for the NOD mouse strain. In addition to lymphocytic infiltrates that develop in the pancreas, the presence of lymphocytic infiltrates in the SMG was reported (5), after which this mouse strain was proposed to serve as a model for Sjögren's syndrome. Other organs of the NOD mouse in which lymphocytic infiltrates have been found include the lacrimal glands and the thyroid (6, 7). In aged NOD mice (> 1 year) that have not developed diabetes, lymphocytic infiltrates can also be detected in the kidney, large intestine, muscle and nervous tissue (8). The incidence of diabetes as well as sialoadenitis in NOD females is higher when compared to males, whereas dacryoadenitis developed more frequently in male mice (9, 10).

The development of insulitis, already apparent in some animals at the age of 4 weeks, occurs before the appearance of lymphocytic infiltrates in the SMG, which can be detected from the age of 8 weeks on (11). The development of sialoadenitis in the NOD mouse is accompanied by a decreased secretory response, a feature not observed in most other mouse models for Sjögren's syndrome, but which is not due to loss of blood glucose regulation in diabetic mice (12, 13).

Several lines of evidence have shown that the two autoimmune processes that develop in the pancreas and the SMG occur independently in the same animal, although the diabetes susceptibility loci *Idd3* and *Idd5* did affect the development of sialoadenitis (14). First, it was shown that neither insulitis nor the presence of islet cells is required for the development of sialoadenitis (15, 16). Second, using congenic NOD.B10.H2b mice, in which the unique NOD MHC I-A^{g7} is replaced by the C57BL/10 derived MHC, it was demonstrated that the NOD I-A^{g7} is an essential locus for the development of diabetes, but not for the development of exocrine gland dysfunction (17). Third, the induction of immunological tolerance against pancreatic ß-cells by intrathymic injection of islet cell homogenates into neonatal mice prevented the development of diabetes, whereas the autoimmune response to the salivary glands was not affected (18). This indicates that the autoimmune reaction to the salivary glands is not due to loss of immunological tolerance for the antigen(s), expressed both by the pancreatic ß-cells and the salivary glands.

Composition of lymphocytic infiltrates

A predominance of $CD4^+$ T lymphocytes over $CD8^+$ T lymphocytes was demonstrated in lymphocytic infiltrates in submandibular and lacrimal glands of NOD mice. B cells were also present, but fewer in number when compared to total T cells (6, 19, 20). A diverse repertoire of TCR Vß usage, with a predominance of TCR Vß8.1,2, Vß6 and Vß4 on lymphocytes infiltrating the SMG was described (19, 21). This suggests that T cells expressing these TCR Vß genes may expand clonally in the salivary glands, and proliferate by antigen driven stimulation. However, evidence has also been raised suggesting that initial infiltration of T lymphocytes into the salivary gland may be antigen driven, which is followed by a secondary influx of T lymphocytes expressing different TCR Vß genes (19).

Cytokines

The development of lymphocytic infiltrates in the SMG coincides with an increased mRNA expression of the cytokines IL-1ß, IL-2, IL-6, IL-7, IL-10, IL-12, IFN-γ and TNF-α, whereas expression of IL-4 was not observed (20, 22, 23). Immunohistochemical stainings revealed expression of IL-2, IL-10, IFN-γ and TNF-α by infiltrating lymphocytes, but not of IL-4 (23). Transgenic expression of a soluble TNF receptor in NOD mice resulted in significantly lower infiltration of the exocrine glands, suggesting an important role for TNF-α in the development of lymphocytic infiltrates in these glands (24). These studies show that $CD4^+$ T lymphocytes in the NOD SMG possess a Th1 cytokine profile in addition to IL-10, which is consistent with observations in patients with Sjögren's syndrome (25-29). In the lacrimal gland of the NOD mouse, in which lymphocytic infiltrates are first detected at 8 weeks of age, mRNA expression of IP-10 and RANTES was found from this age on, peaking at 24 weeks. In addition, minimal expression of lymphotactin was observed throughout the disease course (30). Treatment of NOD mice with an anti-RANTES antibody from 6 till 11 weeks of age resulted in a significant reduction of inflammation in the lacrimal gland, suggesting an important contribution of this chemokine to the development of dacryoadenitis.

Autoantibodies

Autoantibodies, present in the serum of NOD mice include anti-thyroid antibodies, and antibodies directed to pancreatic ß cell antigens (7, 8, 31). By immunohistochemistry on murine parotid and SMG sections, antibodies to acinar and ductal epithelial cells were revealed (32). In a low percentage of NOD mice, antibodies to the 52 kD ribonucleoprotein SS-A/Ro were present whereas antibodies directed to SS-A/Ro 60 kD or SS-B/La were not detected (21). In humans, a 120 kD form of the cytoskeletal protein α-fodrin, which normally has a size of 240 kD, has been implicated in the pathogenesis of Sjögren's syndrome (33). Interestingly, antibodies to 120 kD α-fodrin have been detected in serum of NOD mice, correlating closely with the appearance of lymphocytic infiltrates in the salivary glands (22). A role for autoantibodies directed towards salivary gland epithelial cell surface antigens, such as the M3 type muscarinic receptor, in the effector phase of sialoadenitis in the NOD mouse has been suggested, which will be discussed hereafter.

Abnormalities in the salivary gland

The initiation of an aberrant autoimmune reaction, which could lead to the development of an autoimmune disease may be due to an abnormality in one or both contributors to this reaction, i.e. the immune system and/or the target organ to which the autoimmune reaction is directed. The existence of NOD-*scid* mice which lack functional B and T lymphocytes (34) offers good opportunities to discriminate between contributions to the autoimmune response in the SMG of NOD mice that are dependent on the presence of lymphocytes from those that are not. Evidence has been obtained which suggests the existence of genetically programmed abnormalities in the exocrine glands of NOD mice that may contribute to the initiation of the autoimmune reaction. Biochemical analysis of whole saliva

samples from NOD-*scid* mice revealed a different protein composition when compared with control mice, whereas total salivary flow and protein concentration were comparable between NOD-*scid* and control mice (35). An abnormal isoform of parotid secretory protein (PSP) was detected in the saliva of 20-week-old NOD-*scid* mice, and two other isoforms of this protein disappeared. Furthermore, PSP was ectopically expressed in the SMG of 10-week-old NOD-*scid* mice.

Other aberrances found in the salivary glands of NOD and NOD-*scid* mice (18-20 weeks of age), include increased cysteine protease activity and increased expression of matrix metalloproteinases (36, 37). The fact that these abnormalities were also detected in NOD-*scid* mice indicates that disturbed protein expression by salivary glands of mice with the NOD background is likely to originate in the salivary gland as opposed to be caused by (products of) lymphocytes. Furthermore, increased numbers of apoptotic salivary gland epithelial cells has been observed in 18-week-old NOD and NOD-*scid* mice (38). Altogether, these results indicate altered glandular homeostasis in mice with the NOD genetic background, which may contribute to the development of sialoadenitis.

Defects in the immune system

In addition to abnormalities in the NOD SMG, defects in the immune system of the NOD mouse could also contribute to the initiation of the autoimmune response. Defects that have been described include a decreased ability of NOD antigen-presenting cells to stimulate T suppressor cells, whereas the capacity to activate autoreactive T cells is retained (39, 40). This may be related to the relatively unstable NOD MHC class II antigen, to decreased expression of CD86 on NOD antigen-presenting cells, to decreased production of intracellular glutathione and IL-1 by NOD macrophages, or to enhanced prostanoid metabolism in NOD macrophages (39-43).

Anti-CD3 stimulation of T lymphocytes results in upregulation of both CD28 and CTLA-4. The ratio between these molecules can influence the outcome of this stimulation, since CD28 delivers a positive, and CTLA-4 delivers a negative signal to the T cell (44, 45). Following immune activation of NOD T lymphocytes, the ratio between CTLA-4 and CD28 was not increased, in contrast to what was observed on control T lymphocytes. This can result in the inability to control T cell responses, which may increase susceptibility to the development of autoimmune diseases (42). Recently, aberrant cytokine production by LPS stimulated NOD peritoneal macrophages was demonstrated. The precise balance between IL-10 and TNF-α production following stimulation of NOD macrophages with LPS was disturbed, and IL-12 production exceeded by far that of stimulated control macrophages (46). Elevated IL-12 production by NOD macrophages could result in a bias of T cell responses towards a Th1 phenotype, thereby predisposing to the development of organ-specific autoimmunity. Prolonged immune responses of NOD B- and T-lymphocytes have been described *in vitro* (47, 48). This was linked to increased resistance to apoptosis induction, a phenomenon that segregated with several chromosomal loci, among which the *Idd5* diabetes susceptibility region (49-51). NOD hepatocytes were less sensitive to apoptosis induction by D-galac-

tosamine and TNF-α as compared with C57BL/6 hepatocytes, a difference which was due to a postreceptor defect, as binding of recombinant TNF-α to NOD and control hepatocytes was similar (52). It is possible that this defect is not confined to hepatocytes, but extends to leukocytes. Defective activation of T suppressor cells in combination with increased resistance of NOD lymphocytes to apoptosis may contribute to the development of autoimmunity in the NOD mouse.

Mechanisms involved in the effector phase of the autoimmune process

In the NOD mouse, the effector phase of the autoimmune process is accompanied by a decreased secretory reponse of the exocrine glands. Loss of secretory function was shown to be lymphocyte dependent, since it was not observed in NOD-*scid* mice (35). A role for serum autoantibodies was postulated following the observation that NOD.Igμ^{null} mice, in which B-lymphocytes are absent, maintain normal secretory function despite the development of focal infiltrates. Dryness was induced in these mice by transfer of purified serum IgG from NOD mice, which resulted in loss of stimulated saliva production (15). Similar observations came from experiments in which serum from 6 month-old NOD mice was injected into 6-week-old NOD mice, resulting in a decreased secretory response in the recipients (53). In serum of patients with Sjögren's syndrome, anti-M3 muscarinic receptor autoantibodies had been identified (54). Muscarinic receptors are responsible for generation of the fluid phase of saliva. Transfer of IgG from Sjögren's patients to NOD mice resulted in decreased stimulated saliva production in the recipients. Consequently, it was proposed that autoantibodies directed to the M3 muscarinic receptor also mediate decreased secretory function in the NOD mouse (15). Indeed, infusion of anti-M3 muscarinic receptor antibodies into NOD-*scid* mice resulted in a decreased secretory response, whereas antibodies to the ribonucleoproteins SS-A/Ro and SS-B/La did not. Similar results were obtained when C57BL/6-*scid* mice were used, indicating that it is not the NOD genetic background that predisposes to loss of exocrine function in response to anti-M3 receptor antibodies (55).

A role for apoptosis in the effector phase of sialoadenitis was postulated following the detection of increased numbers of apoptotic epithelial cells in SMG of 18-week-old NOD and NOD-*scid* mice (38). However, in NOD-*scid* mice a decreased secretory response does not develop, despite increased numbers of apoptotic cells, arguing against a contribution of apoptosis to diminished secretory function. Still, it can be hypothesized that the induction of apoptosis results in the activation of proteases that may aberrantly cleave cellular proteins, possibly leading to the generation of cryptic antigens that can activate autoreactive lymphocytes, and contribute to the continuation of the autoimmune response. In this regard, it is of interest to note that generation of 120 kD α-fodrin from 240 kD α-fodrin can be mediated by proteases, activated following induction of apoptosis (56).

Increased gelatinase activity has been demonstrated in saliva and salivary gland lysates of 20-week-old NOD, NOD.B10.H2b, and NOD-*scid* mice. Messenger RNA expression of the gelatinases MMP-2 and MMP-9 was elevated in NOD submandibular and parotid glands when compared with controls (37). Because these enzymes are capable of degrading

extracellular matrix components, which can lead to epithelial cell death, these enzymes may also be involved in the effector phase of the autoimmune process. However, treatment of NOD mice from 7 to 20 weeks of age with a broad spectrum MMP inhibitor did neither stop nor retard the development of autoimmune exocrinopathy (37).

The NOD mouse as a mouse model for Sjögren's syndrome

In the NOD mouse, the development of lymphocytic infiltrates is accompanied by a concomitant, antibody mediated decreased secretory response. An important role for autoantibodies in the loss of secretory function has been demonstrated in patients with Sjögren's syndrome; therefore, the NOD mouse offers good opportunities to study this part of the autoimmune reaction in detail. The availability of the NOD-*scid* mouse enables distinction between lymphocyte and non-lymphocyte mediated abnormalities that develop in the exocrine glands. We hypothesize that intrinsic abnormalities in the salivary gland of the NOD mouse may, together with defects in the immune system of this mouse strain, lead to the development of sialoadenitis. Studies on NOD derived mice in which abnormalities in the SMG or in the immune system are normalized will reveal the importance of both aspects to the autoimmune response.

MRL/lpr mouse

General features

The MRL/lpr (or MRL/l) mouse was originally described to develop a systemic lupus erythematosus (SLE)-like syndrome (57). Abnormal lymphoid proliferation was observed with B cell hyperactivity, the presence of autoantibodies, and circulating immune complexes, leading to immune complex glomerulonephritis. A high incidence of synovial and periarticular inflammation was described, which was shown to be similar to human rheumatoid arthritis (57, 58). Following the demonstration that destructive mononuclear infiltrates develop in the salivary and lacrimal glands of a high percentage of mice, it was proposed that the MRL/lpr mouse could serve as a model for Sjögren's syndrome (58, 59). The incidence of sialoadenitis in this mouse strain does not differ between male and female mice, in contrast to lacrimal gland infiltration, which occurs more often in female when compared with male mice (10). The development of sialoadenitis in the MRL/lpr mouse was not related to the development of arthritis since the incidence of sialoadenitis in arthritic and non-arthritic mice was similar (58).

The MRL/lpr mouse is derived from the MRL/MpJ (also MRL/+, or MRL/n) mouse strain. Both substrains differ only by the presence or absence of the *lpr* (lymphoproliferation) mutation. This mutation was mapped to chromosome 19 and involves a mutation of the extracellular domain of the Fas gene (60). Autoimmunity also develops in MRL/n mice, but the onset of disease manifestations is slower and results in a milder form of disease, indicating that the *lpr* mutation accelerates rather than causes disease (61, 62).

Sialoadenitis in the SMG of the MRL/lpr mouse develops from the age of 2 months

on, when lymphocytic infiltrates are predominantly present around the blood vessels. At 3 months of age, they can be detected around the salivary ducts. Infiltrates also develop in the parotid and sublingual glands, but appear later and are less pronounced when compared with the SMG (63). Focal infiltration in the lacrimal gland of the MRL/lpr mouse is detectable from the age of 1 month on (64). The development of sialoadenitis in MRL/lpr mice was demonstrated to be polygenic. Some genes were in common with those associated with other autoimmune disease phenomena in MRL/lpr mice, whereas others were not (65). In a study in which the background genes that participate in the development of vasculitis, glomerulonephritis, arthritis and sialoadenitis were examined, different background genes were identified for each type of lesion. One region on chromosome 10 was associated with sialoadenitis in both male and female mice, whereas a locus on chromosome 4 correlated with the development of sialoadenitis only in female mice. The region located on chromosome 10 was close to the region associated with vasculitis, suggesting that for these aspects of autoimmune disease in the MRL/lpr mouse strain, a common gene may exist (66). The cellular basis for the development of sialoadenitis in the MRL/lpr mouse was demonstrated in experiments in which transfer of mononuclear cells, isolated from the SMG of MRL/lpr mice, to SCID mice or to 3-week-old MRL/lpr mice resulted in the development of inflammatory lesions in the SCID mice, and in accelerated and more severe sialoadenitis in the MRL/lpr mice (67, 68).

Composition of lymphocytic infiltrates

Several studies have demonstrated a predominance of $CD4^+$ T lymphocytes in the inflammatory infiltrates in the SMG of the MRL/lpr mouse, to the expense of $CD8^+$ T lymphocytes and B lymphocytes (63, 69, 70). A similar picture was observed in the lacrimal gland (71). The infiltrating T lymphocytes were shown to express a diverse TCR Vß repertoire, although TCR Vß4, Vß8.1,2 and Vß10b were predominantly expressed (72). Treatment of MRL/lpr mice for two weeks with a cocktail of antibodies directed to TCR Vß4, Vß8.1,2 and Vß10b resulted in amelioration of established sialoadenitis (73). *In vitro*, treatment of mononuclear cells from SMG of MRL/lpr mice with antibodies to CD4 or Vß8, prior to transfer to SCID mice, prevented the development of sialoadenitis in the recipients (67). These experiments suggest an important contribution of $CD4^+$ T cells, expressing particular TCR Vß genes to the autoimmune process in the salivary glands of the MRL/lpr mouse.

Cytokines

Messenger RNA expression of the cytokines IL-1ß and TNF-α in the SMG of the MRL/lpr mouse was detected at 1 and 2 months of age, whereas IL-6 mRNA expression was detected from the age of 3 months on, when extensive infiltration of the glands had occurred (74). Other cytokines that are expressed in the SMG of the MRL/lpr mouse include IFN-γ, IL-12, and TNF-ß (69, 70, 75). It was suggested that early expression of IL-1ß, TNF-α, and IL-12 could induce IL-6 and IFN-γ expression, which may both exert important, but separate roles in the perpetuation of the autoimmune response. Expression of IL-6 could lead to stimulation of B cells and continued production of (auto)antibodies, whereas IFN-γ could induce

MHC class II expression on glandular epithelial cells, resulting in enhanced presentation of self-antigens and continuation of the autoimmune response (70). In lacrimal glands of the MRL/lpr mouse, increased numbers of IL-4 producing cells as compared to IFN-γ producing cells were detected, which contrasts with the SMG, in which no, or a low level of IL-4 expression was found (70, 71, 75).

The involvement of chemokines in the development of autoimmune sialoadenitis in the MRL/lpr mouse was suggested following observations of MCP-1, MIP-1ß and RANTES mRNA expression in SMG of 3-month-old MRL/lpr mice, whereas MIP-1α was additionally expressed at 5 months of age (76). By immunohistochemistry, MCP-1 producing cells were identified in the vicinity of blood vessels and around lymphocytic infiltrates. The importance of MCP-1 in the development of autoimmunity in the MRL/lpr mouse was demonstrated using MCP-1 deficient MRL/lpr mice. These mice showed increased survival when compared with wild-type mice, with reduced incidence and severity of lymphadenopathy, kidney and lung pathology, and skin lesions (77). Although the salivary glands were not included in this study, it can be imagined that MCP-1, the expression of which has been demonstrated in the SMG of the MRL/lpr mouse (76), also plays an important role in the development of sialoadenitis in this mouse strain.

Autoantibodies

Serological analysis of MRL/lpr mice revealed the presence of increased IgM and IgG levels when compared with control mice. High levels of antinuclear antibodies (ANA) were detected, including anti-dsDNA and anti-ssDNA antibodies, as well as antibodies to glycoprotein gp70, and IgM and IgG rheumatoid factors (61). Autoantibodies to the 52 kD ribonucleoprotein SS-A/Ro were detected in over 30% of MRL/lpr mice at 4-5 months of age, whereas antibodies to 60 kD SS-A/Ro and to SS-B/La were only present in 6% of MRL/lpr mice. Anti-52 kD-SS-A/Ro producing plasma cells were detected not only in spleen and lymph nodes but also in the salivary glands of MRL/lpr mice (78). In another study, antibodies directed to SS-B/La were detected in the serum of 30% of male MRL/lpr mice, aged 3-6 months (79). Serum autoantibodies specific for salivary gland tissue homogenates were exclusively found in MRL/lpr mice aged 2-3 months, and disappeared thereafter (70). MRL/lpr mice, deficient in IFN-γ or IL-4 were established to examine the role of these cytokines in the development of autoimmune disease. Whereas absence of IFN-γ resulted in decreased production of autoantibodies (antinuclear antibodies, anti-dsDNA, anti-snRNPs), absence of IL-4 only resulted in decreased production of IgG_1 and IgE, and levels of autoantibodies were not affected. Both in IFN-γ and IL-4 deficient mice, sialoadenitis did not develop (80).

Abnormalities in the salivary gland

In contrast to the NOD and the NOD-*scid* mouse strains, an MRL/lpr substrain which is devoid of functional B- and T-lymphocytes does not exist. Therefore, it is difficult to discriminate between non-lymphocyte and lymphocyte mediated aberrances in exocrine glands

of this mouse strain. However, studies on SMG of pre-diseased MRL/lpr mice (1 month of age) have revealed elevated expression of IL-1ß and TNF-α mRNA (74). Increased expression of other cytokines and chemokines, of MHC class I and II, as well as an altered response of acinar epithelial cells to stimulation with an α_1-adrenergic agonist are observed following the development of sialoadenitis and thus may reflect a glandular abnormality intrinsic to the SMG, or arise due to the presence of lymphocytic infiltrates.

Defects in the immune system

A major defect in the immune system of MRL/lpr mice is the accumulation of double negative ($CD4^-CD8^-$, but $CD3^+$) T lymphocytes expressing the B cell marker B220 in peripheral lymphoid organs. This is not specific for the MRL/lpr mouse, but can be observed in other mouse strains bearing the *lpr* mutation as well, although in mice without an autoimmune background it is not accompanied by the development of autoimmune disease (81, 82). The double negative T cells are refractory to stimulation with mitogens or antibodies against the CD3-TCR complex. They do not express IL-2 and IL-2 receptors, and are not able to proliferate following stimulation (83, 84). However, it has been demonstrated that double negative T lymphocytes of MRL/lpr mice can exert cytolytic activity when stimulated through the CD3-TCRαβ complex, or when triggered using antibodies against the adhesion molecules CD44 or $gp90^{MEL-14}$ (85). Evidence for a role of double negative T cells in the development of autoimmune disease in the MRL/lpr mouse, however, remains to be established.

Due to defective Fas expression in the MRL/lpr mouse, T lymphocytes cannot undergo Fas-mediated apoptosis. Since this holds true for both autoreactive and non-autoreactive T lymphocytes, this defect may contribute to acceleration of the autoimmune process in MRL/lpr mice. Central T cell tolerance does not appear to be affected by the *lpr* mutation. When sufficient TCR activation has taken place as occurs in the thymus, pathways of apoptosis that are independent of Fas signaling become dominant (86).

A defect that has been described in the immune system of MRL/lpr and MRL/n mice, but also in other autoimmune-prone mouse strains, such as the NZB, NZB/W F1, and the NZW strains, is decreased LPS-induced expression of IL-1α and IL-1ß by peritoneal macrophages (87). Dysregulated expression of IL-1 did already fully manifest at birth, and did not change with time (88). In later studies, defective cytokine production by macrophages from lupus-prone mice was shown not to be limited to IL-1, but to extend to IL-6, IL-12, and TNF-α (89). It was suggested that defective TNF-α production contributes to reduced expression of IL-1 and IL-6, since addition of exogenous TNF-α reduced this defect (90). Dysregulated expression of IL-12 was not restored by addition of TNF-α, whereas exogenous IFN-γ could restore IL-12 production. This suggests that once a Th1 response has started, propagation of the response is not disturbed (89). Using irradiation chimeras, decreased LPS-induced IL-1 production was shown to develop independently of the host environment and thus may reflect an intrinsic macrophage defect (88). When macrophages of lupus-prone mice, including MRL/+ mice, were cultured in FCS-free medium, LPS-induced cytokine pro-

duction was normal (91). It was demonstrated that apoptotic cells, opsonized with delipidated FCS could elicit defective cytokine expression by LPS-stimulated macrophages from MRL/+ mice, whereas nonopsonized apoptotic cells or delipidated FCS alone could not. This indicates that interaction of a non-lipid FCS factor with the surface of apoptotic cells creates a ligand capable of eliciting the defect. In addition, it was shown that the defect extended to more cytokines than the ones mentioned before, and that LPS-induced expression of GM-CSF, MIP-1ß, RANTES, and IL-10 was also affected. Koh *et al* hypothesized that the basis of defective cytokine expression resides in a signaling pathway that is triggered upon recognition or uptake of apoptotic cells by macrophages, but which may affect other macrophage functions as well (91). However, defective cytokine expression alone is not sufficient for the development of autoimmunity since macrophages from mice whose genomes contribute to the development of SLE, but themselves are not victim of the disease, exhibit the dysregulated cytokine expression to the same extent as mice in which full-blown SLE develops. A disturbed cytokine profile may however create an imbalance in the regulatory pathways governing the immune response, resulting in increased susceptibility to the development of an autoimmune reaction.

Mechanisms involved in the effector phase of the autoimmune process

Although the development of sialoadenitis in the MRL/lpr mouse is not accompanied by a concomitant decreased secretory response, extensive infiltration in the salivary glands results in destruction of glandular tissue (59). The preservation of normal secretory function despite the presence of destructive lymphocytic infiltrates may be explained by the fact that MRL/lpr mice have a limited life span, usually in the range of 150 days, glomerulonephritis and renal failure being the most common causes of death (92). Although extensive SMG infiltration can be observed at 5 months of age, this process probably has not yet progressed enough to result in loss of secretory function.

The production of nitric oxide radicals has been implied in the development of autoimmunity in MRL/lpr mice. Enhanced expression of inducible nitric oxide synthase (iNOS) has been demonstrated in MRL/lpr macrophages, liver, kidney and spleen. Furthermore, elevated levels of nitric oxide (NO), complexed to hemoglobin were measured in blood of MRL/lpr mice, in parallel with the development of autoimmunity. When the production of NO radicals was inhibited systemically *in vivo*, the development of glomerulonephritis, arthritis and vasculitis was reduced, whereas levels of anti-DNA autoantibodies and glomerular deposition of immune complexes were not modified (93). Although the effect on sialoadenitis was not examined, a role of NO radicals in the induction of damage to the salivary and lacrimal glands can be envisaged.

Increased sensitivity of SMG acinar epithelial cells to α_1-adrenergic stimulation was demonstrated in 12-week-old MRL/lpr mice when compared with young MRL/lpr mice and MRL/n mice (94). It was suggested that production of IL-1ß and/or IL-6 by salivary gland epithelial cells or by infiltrating lymphocytes could inhibit the release of neurotransmitters *in vivo*. This could result in accumulation of second messengers or upregulation of receptor

expression, and increased responsiveness *in vitro*, a process called denervation supersensitivity. However, although decreased functional activity of acinar epithelial cells may exist *in vivo*, it does not result in diminished salivary output in MRL/lpr mice.

Although induction of apoptosis via Fas/ FasL is disrupted in MRL/lpr mice, apoptosis could still be mediated through other pathways. In the thymus of MRL/lpr mice, similar numbers of apoptotic cells were detected when compared with MRL/n and BALB/c mice (95). In the same study, the presence of apoptotic cells in central parts of epimyoepithelial islands of the SMG was described, demonstrating that apoptosis can occur in the glandular parenchyma of the MRL/lpr mouse. Furthermore, expression of several apoptosis-related proteins has been described in the salivary and lacrimal glands of the MRL/lpr mouse (96). Therefore, a role for apoptosis in the destruction of glandular epithelial cells can not be ruled out.

The MRL/lpr mouse as a mouse model for Sjögren's syndrome

In the MRL/lpr mouse, the development of sialoadenitis and dacryoadenitis is accompanied by SLE and arthritis. The development of an additional autoimmune disease has also occurred in patients with secondary Sjögren's syndrome, which makes this mouse strain suitable for comparison with this subgroup of patients, but does not exclude comparison with primary Sjögren's patients. Although an MRL/lpr mouse strain which is devoid of T and B lymphocytes does not exist, the process of sialoadenitis development lasts sufficiently long to study the salivary glands both prior to and following the development of lymphocytic infiltrates.

Different features of Sjögren's syndrome in MRL/lpr mice, NOD mice and humans are summarized in Table 1. From this table it can be concluded that MRL/lpr and NOD mice share specific disease characteristics with Sjögren's syndrome as it develops in humans. In the MRL/lpr mouse model, the development of sialoadenitis and dacryoadenitis is associated with systemic autoimmune phenomena and is not accompanied by decreased exocrine secretion. The NOD mouse, on the other hand, exhibits characteristics of organ specific autoimmunity, and autoantibodies are thought to mediate decreased production of saliva and tears. In addition to the differences observed in the effector phase of the autoimmune reaction, the origin of the event(s), leading to the initiation of the autoimmune reaction may differ between the two mouse models, although this is still hypothetical.

NZB/W F1 mouse

F1 female hybrids of New Zealand Black and New Zealand White (NZB/W F1) mice spontaneously develop lymphocytic infiltrates in the salivary and lacrimal glands in addition to hypergammaglobulinemia, autoantibodies and immune-complex mediated glomerulonephritis (97, 98). The lesions in the salivary and lacrimal glands can be observed from 4 months of age on, and are more severe in female than in male mice. In NZB mice, lymphocytic infiltration in the salivary and lacrimal glands was also described, less extensive than in NZB/W F1 mice, whereas NZW mice did not show signs of sialoadenitis or dacryoadenitis

Table 1. Features of Sjögren's syndrome in NOD mice, MRL/lpr mice and humans

	NOD mouse	MRL/lpr mouse	Humans
Sex distribution	sialoadenitis F > M dacryoadenitis M > F	sialoadenitis F = M dacryoadenitis F > M	F > M
Involvement of other organs	pancreas thyroid gland kidney large intestine muscle nervous tissue	kidney joints blood vessels	kidney bladder stomach liver pancreas thyroid gland respiratory system heart blood vessels neural tissue
Composition of lymphocytic infiltrates	SMG: $CD4^+$ T cells > $CD8^+$ T cells > B cells	SMG: $CD4^+$ T cells > $CD8^+$ T cells, B cells	minor salivary glands: $CD4^+$ T cells > $CD8^+$ T cells T cells > B cells
Autoantibodies	anti-thyroid Ab Ab to ß-cell antigens (anti-SS-A/Ro 52 kD) anti-120 kD α-fodrin Ab anti-ß adrenergic R Ab anti-M3 muscarinic R Ab	anti-ss DNA Ab anti-ds DNA Ab anti-gp70 Ab rheumatoid factor (RF) anti-SS-A/Ro 52 kD (anti-SS-A/Ro 60 kD) anti-SS-B/La	anti-SS-A/Ro 52 kD anti-SS-A/Ro 60 kD anti-SS-B/La RF anti-120 kD α-fodrin Ab anti-M3 muscarinic R Ab anti-M1 muscarinic R Ab
Loss of secretory function	yes	no	yes

(98). Lymphocytic infiltrates in the SMG predominantly contained $CD4^+$ T cells, while $CD8^+$ T cells and B lymphocytes were also detected (99).

Cellular immune functions of NZB/W F1 mice decreased with age, a phenomenon that developed concurrently with autoimmunity in this mouse strain, and which was not observed in control mice. It was suggested that decreased immune function may preferentially affect cells that normally suppress immune responses to a variety of antigens, perhaps including autoantigens, and in this way contribute to the development of autoimmunity (100). Macrophages from NZW mice show underexpression of IL-1 when stimulated with LPS, to a similar degree as macrophages from MRL/lpr mice (87). The fact that NZW mice are phenotypically normal indicates that IL-1 underexpression alone is not sufficient to result in the development of autoimmune disease in this mouse strain.

The effect of dietary calorie restriction on the development of sialoadenitis NZB/W F1 mice has been studied. Although no significant effects were observed in 3.5-month-old mice, the inflammatory process in the SMG of 8.5-month-old mice was significantly reduced as compared with mice, fed *ad libitum* (101). Calorie restriction was accompanied with increased levels of TGF-ß1 mRNA, whereas IL-6 mRNA and TNF-α mRNA were significantly increased in *ad libitum* fed NZB/W mice. It was suggested that increased expression

of TGF-ß1 could lead to decreased recruitment to and immunosuppression of inflammatory mononuclear cells in the salivary glands. However, the mechanism responsible for increased TGF-ß1 expression in calorie restricted mice was not revealed.

In conclusion, autoimmune phenomena developing in NZB/W F1 mice, may be comparable to MRL/n and MRL/lpr mice, although in MRL/n mice disease progression (especially glomerulonephritis) is less severe and results in lower mortality when compared with NZB/W F1 and MRL/lpr mice. Observations in NZB/W mice can be compared with the NZB and NZW parental strains, in order to assess the factors that contribute to the development of autoimmune disease in this mouse model. Comparison of NZB/W F1 mice and NZB mice on the one hand with NZW mice on the other hand may provide clues to events that play a role in the initiation of sialoadenitis in this mouse strain. Factors which differ between NZB and NZB/W F1 mice may account for the perpetuation of the autoimmune reaction in NZB/W F1 mice.

TGF-ß1 knockout mouse

Targeted disruption of the gene coding for the immunomodulatory cytokine TGF-ß1 resulted in the establishment of a mouse strain that shows no gross developmental abnormalities, but die by three weeks of age. Cause of death is severe wasting syndrome, with inflammatory cell infiltration in multiple organs, including the heart, lung, pancreas, and salivary glands (102, 103). The infiltrates in the salivary glands were shown to be periductal, predominantly containing $CD4^+$ T lymphocytes (104). Early accumulation of lymphocytes around ducts in the SMG can be observed at 1 week of age. The development of lymphocytic infiltrates resulted in disruption of salivary gland architecture. Acinar epithelial cells in the vicinity of lymphocytic infiltrates were atrophic and produced less mucin when compared with non affected acinar cells (105). In addition to the salivary glands, the lacrimal glands were severely affected, with crusty deposits developing around the eyes. Lesions in the lacrimal glands were composed of T cells, B cells and limited numbers of macrophages (106). In the sera of TGF-ß1 knockout mice autoantibodies directed to dsDNA, ssDNA, and Sm ribonocleoprotein were detected, whereas antibodies to SS-A/Ro, SS-B/La, and rheumatoid factor were absent. The presence of these autoantibodies led to the deposition of immune complexes in the glomeruli as well as in the parenchyma of the SMG (105, 107).

Increased expression of IL-1ß and TNF-α mRNA was observed in the SMG of TGF-ß1 knockout mice when compared with controls, whereas IL-6 mRNA was exclusively expressed in glands of knockout mice (105). Other cytokines that were highly expressed in the salivary gland include IL-1α, IL-2, IL-4, IL-10, and IFN-γ (104). Adhesion molecules were suggested to play an essential role in the development of sialoadenitis and lacrimal gland pathology in this model, since administration of synthetic fibronectin peptides could block the development of lymphocytic infiltrates and accompanying changes in glandular physiology (105, 106). A decreased stimulated saliva production can be observed in TGF-ß1 knockout mice, from 18 days of age on. This was suggested to affect the nutritional state of the mice, ultimately contributing to the wasting syndrome. Indeed, when the mice were sup-

plied with liquid diet, the survival was almost twice as long as when regular chow was given (105).

In TGF-ß1/MHC class II double knockout mice, the development of inflammatory lesions in the salivary glands, heart, lung, and liver, as well as the production of autoantibodies to dsDNA, ssDNA and Sm ribonucleoprotein does not occur (104, 108). This demonstrates the dependence on MHC class II antigens for the expression of the autoimmune phenotype in TGF-ß1 deficient mice. A mechanism, explaining the development of autoimmunity in this mouse strain was proposed, involving the presence of continuously activated T lymphocytes that undergo adhesion molecule mediated transmigration through endothelium. In the tissues, MHC class II is highly expressed in cells that can function as nonprofessional antigen presenting cells. This may lead to clonal expansion and proliferation of T lymphocytes, continued upregulation of adhesion molecules and MHC class II molecules, and aggravation of the inflammatory process in the organs which are affected by the autoimmune process (104). In minor salivary glands of patients with Sjögren's syndrome, expression of TGF-ß negatively correlated with severity of sialoadenitis (29), and similar mechanisms may play a role in the perpetuation of sialoadenitis in humans. In patients with Sjögren's syndrome, salivary gland epithelial cells, expressing high levels of HLA-DR molecules, have been suggested to actively participate in the development of sialoadenitis (109, 110). Since autoimmunity in TGF-ß1 deficient mice has been shown to rely on the expression of MHC class II molecules (104, 108), it would be of interest to specifically target MHC class II molecules on antigen presenting cells or on salivary gland epithelial cells, to assess the contribution of both types of cells to the development of sialoadenitis. However, the fast course of the autoimmune phenomena, leading to early death, complicates studies on the salivary glands in pre-diseased mice, as well as studies on perpetuation of the autoimmune response.

3d-Tx NFS/*sld* mouse

A spontaneous autosomal recessive mutation in the nonautoimmune NFS/N mouse, affecting the differentiation of the sublingual gland, resulted in the establishment of the NFS/*sld* mouse strain (111). Whereas no abnormalities were found in the parotid and SMG of NFS/*sld* mice, thymectomy at 3 days of age (3d-Tx NFS/*sld*) resulted in the development of severe inflammatory lesions in the submandibular, parotid, and lacrimal glands from the age of 4 weeks on, but not in the sublingual gland (112). The infiltrates in the salivary glands were periductal, aggravated with increasing age, and led to destruction of parenchymal tissue. The highest incidence of sialoadenitis and dacryoadenitis was observed in female mice. The development of lymphocytic infiltrates (mainly $CD4^+$ T lymphocytes, with smaller numbers of $CD8^+$ T cells and B cells) was accompanied by the presence of serum autoantibodies directed to salivary duct epithelial cells (112). At 4 weeks of age, cells present within the inflammatory infiltrates expressed IL-2, IFN-γ, TNF-α, and sometimes IL-10, whereas IL-6 positive cells were detected at 12 weeks of age. In SMG of 3-week-old mice with few infiltrating cells, mRNA expression of IL-1ß, TNF-α, IL-2, IFN-γ, IL-10 and IL-12 was found. Local upregulation of cytokine and adhesion molecule expression was suggested to

play a role in the initiation of the autoimmune reaction in this mouse model (113).

Between 1 and 3 weeks of age, a unique $CD4^+$ T cell subset expressing low levels of CD28 was detected in the spleen of 3d-Tx NFS/*sld* mice, which disappeared following disease onset. These cells expressed regulatory cytokines (IL-4, IL-10, and TGF-ß), and transfer of this T cell subset was shown to ameliorate the development of autoimmune lesions in the salivary and lacrimal glands. Disappearance of $CD4^+CD28^{low}$ T cells following disease onset may be due to release of autoantigen and activation of these cells, resulting in upregulation of CD28 expression (114).

The 3d-Tx NFS/*sld* mouse model for Sjögren's syndrome is especially interesting since it was used in the identification of 120 kD α-fodrin as an autoantigen in Sjögren's syndrome (33). This autoantigen was purified from salivary glands of 3d-Tx NFS/*sld* mice, and sera of these mice contained autoantibodies directed to 120 kD α-fodrin. In addition, splenic T lymphocytes showed a proliferative response to the protein. Intravenous injection of a recombinant protein, matching the aminoterminal portion of α-fodrin, was able to inhibit the development of sialoadenitis in this mouse model, demonstrating the involvement of this cytoskeletal protein in the initiation of sialoadenitis. The mechanism, responsible for the generation of 120 kD α-fodrin, was suggested to be apoptosis, resulting in activation of proteases and cleavage of the 240 kD form of the protein (33).

When aged (18-20 months old) 3d-Tx NFS/*sld* mice were compared with younger mice (2-4 months old), a decreased secretory response of the salivary and lacrimal glands was observed. An increased number of apoptotic epithelial duct cells as well as an increased proportion of Fas expressing cells was detected in the salivary glands of aged mice, whereas FasL expression on infiltrating $CD4^+$ T lymphocytes did not change with age. High numbers of apoptotic cells in glands of aged mice correlated with augmented levels of 120 kD α-fodrin in the salivary glands as well as with a rise in autoantibody production against this antigen (115).

The necessity of costimulation in the development of sialoadenitis was also studied using 3d-Tx NFS/*sld* mice. Although blockage of CD80 mediated co-stimulation of T lymphocytes resulted in a non-significant reduction in inflammatory lesions, administration of anti-CD86 monoclonal antibodies resulted in a significant suppression of autoimmune phenomena in both the salivary and lacrimal glands. This was associated with a shift towards a Th2 profile of splenic T lymphocytes, decreased proliferative response of splenic cells to 120 kD α-fodrin, and decreased serum levels of autoantibodies to 120 kD α-fodrin (116).

The possible involvement of 120 kD α-fodrin both in the pathogenesis of sialoadenitis and dacryoadenitis in this mouse strain and in the development of Sjögren's syndrome in humans may provide an important clue for the mechanism through which apoptosis contributes to the pathogenesis of Sjögren's syndrome. This mechanism would involve the activation of proteases following induction of apoptosis, which can subsequently cleave cellular proteins, resulting in the generation of neoantigens which can play a role in the initiation or perpetuation of the autoimmune reaction. Since this is based on circumstantial evidence, it needs to be confirmed by additional experiments. The 3d-Tx NFS/*sld* mouse is especially

suited to examine this mechanism in detail.

IQI/Jic mouse

IQI/Jic mice, established from ICR mice, were only recently described as a mouse model for Sjögren's syndrome (117). Sialoadenitis develops in the submandibular and parotid glands of this mouse strain, in addition to dacryoadenitis. In SMG of female mice below 6 months of age, only slight lesions are present, while extensive infiltrates and destruction of acinar tissue was detected from the age of 9 months on. In male mice, only slight and stable lesions develop, the incidence of which increases with age. The main lymphoid cells in small foci are $CD4^+$ T lymphocytes, whereas in large foci mainly B cells are present, followed by $CD4^+$ T lymphocytes. Few $CD8^+$ T lymphocytes as well as some macrophages were detected. At 15 months of age, 33% of female mice exhibited antinuclear antibodies, but no anti-SS-A/Ro, anti-SS-B/La, or anti-salivary gland antibodies were detected (117).

The development of sialoadenitis and dacryoadenitis in IQI/Jic mice occurs at a relatively high age. On the one hand, this results in the IQI/Jic strain being economically unattractive for extensive studies on the pathogenesis of Sjögren's syndrome, while on the other hand it is a unique feature for this mouse strain very much resembling the development of Sjögren's syndrome in humans.

Aly/aly mouse

A spontaneous autosomal recessive mutation in mice, leading to absence of peripheral lymph nodes, Peyer's patches, and disrupted architecture of spleen and thymus originally occurred in the C57BL/6J mouse strain. The mutation was maintained in a hybrid strain derived from C57BL/6J×AEJ/GnRk mice (118). The alymphoplasia (*aly*) mutation was recently mapped to the gene, coding for NF-κB-inducing kinase (Nik), giving rise to an amino acid substitution in the C-terminal region of Nik (119). Homozygotes for the *aly* mutation were deficient in both humoral and cellular immune functions, as demonstrated by severely depressed levels of serum IgA, IgG, and IgM, and the inability to reject histoincompatible skin allografts. B cell maturation in the bone marrow as well as B cell function in the periphery were reduced, whereas splenic T cells produced 50% less IL-2 following anti-CD3 stimulation when compared with T cells derived from *aly/+* mice (120). Furthermore, *aly/aly* lymphocytes have a defect in their migratory capacity *in vivo*, and their *in vitro* chemotactic responses to secondary lymphoid tissue chemokine (SLC) and B lymphocyte chemoattractant (BLC) are impaired. This was shown to be due to defective signal transduction downstream of receptor ligation (121).

From 14 weeks of age on, lymphocytic infiltrates, almost exclusively composed of $CD4^+$ T lymphocytes, can be detected in multiple organs of male and female *aly/aly* mice, including the salivary and lacrimal glands, and the exocrine pancreas. In the lacrimal gland and the pancreas, the development of inflammatory lesions was accompanied with damage to glandular tissue. Autoantibodies to exocrine organs or nuclear components were not detected in this mouse strain (122). Analysis of TCR Vß usage of salivary gland infiltrating lym-

phocytes revealed predominant expression of TCR Vß1 and Vß5 at 15 weeks of age, while expansion of TCR Vß usage was observed therafter (123).

Since mononuclear cell infiltration was occasionally observed in the lung and kidney, in addition to the exocrine glands, the *aly/aly* mouse was suggested to serve as a good model for Sjögren's syndrome, in which pneumonitis, pancreatitis and interstitial nephritis can develop as well (124). However, the widespread immunological abnormalities, present in this mouse strain due to the *aly* mutation complicate extrapolation of possible mechanisms leading to the development of sialoadenitis to humans.

Murine transplantation chimeras (GVHD)

Sialoadenitis in murine transplantation chimeras does not develop in one particular mouse strain. Instead it involves the development of autoimmune disease accompanying chronic graft-vs-host disease (GVHD) in non-irradiated hybrid mice, transplanted with parental spleen cells. In most of these mice, lupus-like disease develops and the presence of anti-nuclear antibodies and anti-dsDNA autoantibodies of IgG, IgM, and IgA isotypes can be detected (125, 126). Severity and time of onset of disease vary, depending on the strains used for the generation of the hybrid, and on the donor mouse strain. In (C57BL/6×DBA/2) F1 mice that received spleen cells from DBA/2 mice, a destructive type of sialoadenitis develops. Epithelial cells in close proximity to lymphocytes appear damaged and display features of cell degeneration. $CD4^+$ T lymphocytes were shown to be the main component of the inflammatory infiltrates, while $CD8^+$ T cells, few B cells, and macrophages were also present (125). In (DBA/2×C3H) F1 recipients of DBA/2 spleen cells, a non-destructive exocrinopathy developed with similar incidence in male and female mice (126). (BALB/c×CBA/H-T6) F1 hybrids that received BALB/c splenic cells did not show typical symptoms of GVHD, but a transient reaction could not be excluded. Sjögren's-like glandular changes developed in this model in the absence of clinical symptoms of SLE, although serum anti-nuclear and anti-dsDNA autoantibodies did occur (127).

It was suggested that autoimmune phenomena in these murine transplantation chimeras are initiated by immune responses of alloreactive donor helper T cells to MHC class II on F1 B cells, leading to polyclonal B cell activation and production of autoantibodies (125). The use of transplantation chimeras in combination with gene knockout or transgenic mice as parental or donor strain, offers the opportunity to study the contribution of separate genes to the development of autoimmunity in this model, an approach that has been used by several groups (128, 129).

Sjögren's-like disease also develops in a significant percentage of patients suffering from chronic GVHD following bone marrow transplantation (130). Comparison of minor salivary glands from these patients with glands from patients with Sjögren's syndrome revealed that relative contributions of different lymphocyte subsets to the infiltrates and expression of HLA-DR and adhesion molecules were different among the two groups (131). This indicates that the pathogenesis of Sjögren's-like disease may be very different from Sjögren's syndrome. With this in mind, transplantation chimeras may better serve as a model

for Sjögren's-like disease accompanying GVHD instead of being used as a model for Sjögren's syndrome.

In Table 2, an overview of characteristics of sialoadenitis is given for mouse models for Sjögren's syndrome which are less often studied for this purpose than the NOD and MRL/lpr mice. From this table it is clear that the onset of disease varies considerably among the different mouse strains. Furthermore, the secretory function, an important hallmark of the disease, has so far not been studied in a significant number of models.

Concluding remarks

A number of mouse strains can be used to study the pathogenesis of Sjögren's syndrome. The perfect mouse model in which all disease manifestations can be studied, does not exist. Instead, each of the mouse models exhibits model specific characteristics that can be compared with a subgroup or with the majority of patients, and which can be exploited to study a particular aspect of the pathogenesis. Therefore, the choice of the mouse model(s) should be related to the particular aspect of the disease and on the phase of the pathogenetic process to be studied. Furthermore, the use of different mouse strains enables investigators to get insight into different pathogenetic mechanisms important in the initiation or in the effector phase of the autoimmune disease.

Table 2. Characteristics of mouse models for Sjögren's syndrome*

	NZB/W F1 mouse	TGF-ß1 knockout mouse	3d-Tx NFS/sld mouse	QI/Jic mouse	Aly/aly mouse	Murine trans-plantation chimeras
Age	4 months	1 week (death by 3 weeks)	4 weeks	6-9 months	14 weeks	varies, depending on the strains used
Sex distribution	F > M	n.d.	F > M	F > M	F = M	F = M
Composition of lymphocytic infiltrates in SMG	mainly $CD4^+$ T cells	mainly $CD4^+$ T cells	mainly $CD4^+$ T cells	small foci: mainly $CD4^+$ T cells, large foci mainly B cells	mainly $CD4^+$ T cells	varies, depending on the strains used
Loss of secretory function	n.d.	yes	yes, 18-20 month old mice	n.d.	n.d.	n.d.

*: Characteristics of NOD and MRL/lpr mice are summarized in table 1.
n.d.: not determined.

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Chapter 1.3

AIM AND OUTLINE OF THIS THESIS

Aim and outline of the studies

The cause of an excessive autoimmune reaction resulting in an autoimmune disease is most likely multifactorial, involving disturbances in the immune system and in the organ(s) to which the reaction is directed. In patients with Sjögren's syndrome, autoimmune phenomena have been described in epithelial compartments of the salivary and lacrimal glands as well as of other exocrine organs. It has been suggested that the primary affected tissue in Sjögren's syndrome is the glandular epithelium. The epithelial cells of the exocrine glands were even proposed to have an active role in the development of Sjögren's syndrome. As outlined in chapter 1.2, studies in the NOD and NOD-*scid* mouse models for Sjögren's syndrome suggest the existence genetically programmed abnormalities in the salivary glands. The purpose of the studies described in this thesis was to increase insight into the role of the cellular constituents of the salivary glands in the initiation of the autoimmune reaction, leading to the development of Sjögren's syndrome. These studies were mainly performed in mouse models for Sjögren's syndrome, in which the salivary glands can be studied in detail from birth onwards until the autoimmune process has fully developed. In addition, human labial MSG were studied. However, as these MSG were mainly collected from patients suspected of having Sjögren's syndrome, the initiation phase of the autoimmune reaction could not be studied in these samples.

In chapter 2, the histopathological development of sialoadenitis in two different mouse models for Sjögren's syndrome, the NOD and the MRL/lpr mouse, are described and compared. In addition, the presence of dendritic cells before and after the development of the lymphocytic infiltrates was examined. Emphasis was put on the dendritic cells as these cells are the most potent antigen presenting cells, capable of activating naïve T lymphocytes. It has been postulated that these cells play a central role in the early phase of several autoimmune diseases.

Studies on the presence of leukocytes in MSG of patients with Sjögren's syndrome have mainly focussed on the presence of lymphocyte subsets in the focal infiltrates, whereas the presence of professional antigen presenting cells, such as dendritic cells and macrophages has received little attention. We studied the presence of subsets of dendritic cells and macrophages in labial MSG of patients with Sjögren's syndrome, controls, and patients with focal lymphocytic sialoadenitis (FLS) or keratoconjunctivitis sicca (KCS). The latter two patient groups fulfilled either the oral or the ocular hallmarks of Sjögren's syndrome. The objective of this study, which is described in chapter 3, was to examine if dendritic cells or macrophages form a component of the non-diseased and diseased salivary glands, and consequently could be involved in the initiation or perpetuation of the autoimmune disease. In addition, we examined if the diffuse presence of these cells in diseased MSG could be of help in the histopathological diagnosis of Sjögren's syndrome.

Apoptosis is mainly regarded as a mechanism of cell death, not inducing an immune response. Nevertheless, evidence is accumulating suggesting that enzymes, activated follow-

ing induction of apoptosis, may cleave cellular proteins, resulting in the generation of cryptic autoantigens and in the initiation of an autoimmune response. Furthermore, apoptosis can lead to the release of antigens normally present within the cell, which may trigger the autoimmune reaction. For this reason, we also examined the presence of apoptotic cells in submandibular glands of NOD and NOD-*scid* mice prior to the development of sialoadenitis. Because apoptosis has been suggested to induce cell damage in the effector phase of autoimmune diseases, including Sjögren's syndrome, numbers of apoptotic cells were also studied in submandibular glands of NOD mice following the development of lymphocytic infiltrates. Furthermore, the expression of the apoptosis related proteins Fas, FasL, and bcl-2 was investigated. The results of these experiments are described in chapter 4.

The objective of the studies described in chapter 5 was to examine if abnormalities occur in the SMG of neonatal NOD and NOD-*scid* mice with regard to glandular morphology, expression of extracellular matrix degrading enzymes, and of extracellular matrix components. Because glandular morphology and morphodifferentiation occurring early in life are influenced by apoptosis of epithelial cells, this process, and the expression of Fas, FasL and bcl-2 were studied as well. Furthermore, the effect of the diabetes susceptibility loci *Idd3* and *Idd5*, which have been shown to influence the development of sialoadenitis, was examined on morphologic differentiation and expression of extracellular matrix components and extracellular matrix degrading enzymes.

Although originally identified as factors produced by lymphocytes and other leukocytes, cytokines and chemokines can also be produced by epithelial cells. Aberrant epithelial expression of these factors could result in the attraction and activation of leukocytes, among which professional antigen presenting cells. These may, after uptake of salivary gland derived antigen, travel to the draining lymph node, and activate autoreactive lymphocytes. For this reason, the expression of proinflammatory cytokines as well as chemokines was studied in SMG of NOD and NOD-*scid* mice before and after the onset of sialoadenitis. The development of lymphocytic infiltrates is likely to influence the expression of chemotactic factors, which may subsequently result in the attraction of additional leukocytes. For this reason, submandibular glands in which lymphocytic infiltrates had developed were also included. These experiments are described in chapter 6.

In chapter 7, the implications of the results as well as suggestions for future studies are discussed.

Chapter 2

TWO DIFFERENT TYPES OF SIALOADENITIS IN THE NOD- AND MRL/lpr MOUSE MODELS FOR SJÖGREN'S SYNDROME: A DIFFERENTIAL ROLE FOR DENDRITIC CELLS IN THE INITIATION OF SIALOADENITIS?

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Abstract

Sjögren's syndrome is an autoimmune disease that primarily affects the salivary and lacrimal glands. In these glands, focal lymphocytic infiltrates develop. Little is known about the initiation of this autoimmune disease. Antigen presenting cells (APC) such as dendritic cells (DC) can play a role in the initiation of autoimmunity. To date, no data on the presence of DC in Sjögren's syndrome are available.

Several mouse strains, the nonobese diabetic (NOD) and the MRL/lpr mouse, can be used as models for Sjögren's syndrome. We compared the development of sialoadenitis in submandibular glands (SMG) of NOD and MRL/lpr mice with particular focus on the presence of APC. DC, macrophages, T cells and B cells in the SMG were studied by means of immunohistochemistry, after which positively stained cells were quantified. NOD-severe combined immunodeficiency (SCID) mice were used to study the presence of APC in the SMG in the absence of lymphocytes.

Before lymphocytic infiltration, increased numbers of DC were detected in the SMG of NOD mice compared with those numbers in control mice and MRL/lpr mice, which suggests that DC play a role in the initiation of sialoadenitis in NOD mice. In the SMG of NOD mice, lymphocytic infiltrates organized in time. In MRL/lpr mice, however, lymphocytic infiltrates were already organized at the time of appearance. This organization was lost in time.

In conclusion, two types of sialoadenitis are described in two mouse models for Sjögren's syndrome. Differences exist with regard to early events that may lead to the development of sialoadenitis and to the composition and organization of inflammatory infiltrates. It is possible that different types of sialoadenitis also exist in humans and that the pathogenetic process in both the early and late phases of the autoimmune reaction differs among patients.

Introduction

Sjögren's syndrome is a systemic autoimmune disease with a chronic course that is characterized by lymphocytic infiltration and destruction of the salivary and lacrimal glands, which causes dryness of the mouth and eyes (1). A combination of immunologic, genetic, hormonal, and possibly also viral factors play a role in the development of this multifactorial disease, but little is known about the early stages of Sjögren's syndrome that lead to the initiation of the autoimmune process (2, 3).

Several mouse models are used to study the pathogenesis of Sjögren's syndrome. Two of those models, the nonobese diabetic (NOD) mouse and the MRL/lpr mouse, are widely accepted (4-6). In both mouse strains, perivascular and periductal lymphocytic infiltrates in the salivary and lacrimal glands are histologic hallmarks of the disease. In NOD mice (not in

MRL/lpr mice) the development of focal lymphocytic infiltrates in the salivary glands (sialoadenitis) is accompanied by a corresponding loss of the secretory function and changes in the protein composition of the saliva (6, 7). These changes in protein composition have also been observed in NOD-SCID (severe combined immunodeficiency) mice (8). The NOD-SCID mouse is devoid of functional T lymphocytes and B lymphocytes because of a homozygosity in the SCID mutation (9). Hence the salivary gland abnormalities in this mouse strain must be considered as the cause rather than a consequence of the focal lymphocytic infiltration. In MRL/lpr mice, the systemic autoimmune response is accelerated compared with that in congenic MRL/Mp mice because of a mutation in the *Fas* apoptosis gene. This results in the failure of autoreactive lymphocytes to be deleted by means of apoptosis and causes an accumulation of these lymphocytes in the periphery (10, 11).

With regard to the composition of the inflammatory infiltrates in the salivary glands of NOD mice, MRL/lpr mice, and patients with Sjögren's syndrome, attention has been focussed on the presence of various subsets of T cells and B cells. Most infiltrating lymphocytes are TCR$\alpha\beta^+$, $CD4^+$ T cells, whereas $CD8^+$ T cells and B cells are fewer in number (12-14). The development of sialoadenitis in the NOD- and the MRL/lpr mouse models for Sjögren's syndrome has never been compared over time.

For the activation of naive T cells, antigen from antigen presenting cells (APC) such as dendritic cells (DC) must be present. In contrast to macrophages, mature DC have limited phagocytic activity but must constitutively express high levels of MHC-class II and costimulatory molecules. Mature DC are the most potent regulators of the immune response (15, 16). Information on the topographic distribution of DC and macrophages before and during the development of sialoadenitis is scarce. In animal models of insulin-dependent diabetes mellitus and of autoimmune thyroiditis, DC and macrophages are the first cells of hematopoetic origin that infiltrate the target organ (17-21). The presence of these cells in the earliest stages of the disease suggests that they play an essential role in the initiation and regulation of the autoimmune reaction.

In this study, immunohistochemical techniques were used to examine the development of sialoadenitis, particularly with respect to the topographic distribution of $CD11c^+$ DC and $BM8^+$ macrophages and the composition of the lymphocytic infiltrates, in the submandibular glands (SMG) of NOD mice and MRL/lpr mice from 2 to 20 weeks of age.

Materials and methods

Mice and experimental design

Female NOD, NOD-*scid*, C57BL/10 and BALB/c mice were bred in our own facilities under specific pathogen-free conditions. Female MRL/lpr mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice (n = 4-8 mice/age/strain) were fed standard pellets and water *ad libitum* and maintained at 22 +/- 1°C on a 12h light / 12h dark cycle. Under these conditions the incidence of diabetes in NOD mice at 30 weeks of age was 90%

in females and 30% in males.

Tissue preparation

At the age of 1-6, 8, 10, 15 and 20 weeks, the mice were killed by asphyxiation with carbon dioxide. The SMG were removed, embedded in Tissue-tek (Sakura Finetek, Torrance, CA) and snap-frozen in liquid nitrogen. Tissues were stored at -80°C.

Immunohistochemistry

Before sectioning, microscopic slides were treated with 95% ethanol/ 5% diethylether solution for 10 minutes, and coated with a solution of 0.1% gelatine with 0.01% chromiumalum in distilled water. Thereafter, 6 µm sections of the frozen tissue specimens were cut, and fixed for two minutes. For this fixation, 0.4 g of pararosanilin (Sigma, St.Louis, MO) was dissolved in 10 ml of 2 M HCl by gently heating to 37°C for 4 h; this was subsequently filtered and stored at 4°C. Of this stock solution, 500 µl was incubated with 4% $NaNO_2$ for 1 minute. This was then added to 165 ml of distilled water in which the slides were fixed. Following the fixation, the slides were rinsed in phosphate-buffered saline (PBS) (pH 7.8) /Tween (0.1 %), after which they were incubated for one hour with the monoclonal antibody N418 (36), KT3 (37), B220 (38) or BM8 (39). Except for BM8 (BMA, Biomedical AG, Augst, Switzerland), all monoclonal antibodies were hybridoma culture supernatants reacting with DC, total T cells, and B cells, respectively. As a negative control for these antibodies, the culture supernatant of cell line Y3, the fusion partner which was used for the generation of the hybridoma cell line was used. BM8, reacting with mature macrophages, was diluted 1:40 in PBS/Tween. After the incubation period, the slides were washed with PBS/Tween, and incubated for 40 minutes with as second antibody a horseradish peroxidase (HRP)-conjugated goat anti-armenian hamster IgG in case N418 was used as primary antibody (Jackson, ImmunoResearch Laboratories, West Grove, PA). This antibody was diluted 1:100 in PBS/Tween to which 2% normal mouse serum was added to reduce background staining. In case KT3, B220, or BM8 was used as primary antibody, the slides were incubated for 40 minutes with HRP-conjugated rabbit anti-rat IgG (DAKO, Glostrup, Denmark) as second antibody. Also this antibody was diluted 1:100 in PBS/Tween to which 2% normal mouse serum was added. After washing in PBS/Tween, the peroxidase label was developed by exposure to 0.10% (wt/vol) di-amino-benzidine (DAB) in acetate buffer (pH 6.0) containing 1% $NiSO_4$ (wt/vol) and 0.02% H_2O_2 for 3 minutes. The slides were washed in PBS/Tween, counterstained by nuclear fast red (0.1% (wt/vol) solution in water, containing 5% (wt/vol) $Al_2(SO_4)_3$), dehydrated by an ethanol/xylene series and embedded with Depex mounting medium (BDH, Poole, England).

Image analysis

For quantitative analysis of sections the VIDAS-RT image analysis system (Kontron Elektronik GmbH/Carl Zeiss, Weesp, The Netherlands) was used. All sections were analyzed by two independent individuals. Enumeration of cells expressing the N418 cell surface mark-

er was performed as follows: At a magnification of 100, 5 areas of the microscopic slides, which were located outside lymphocytic infiltrates, were counted. No part of a lymphocytic infiltrate was present in these areas. One area consisted of 262,144 pixels, the surface area of 10,000 pixels being 11.326 μm^2. After image capture, background color was excluded by image thresholding, after which the diaminobenzidine reaction product was quantified. Results were expressed as the mean number of positive pixels per area. The percentage of the total area of the lymphocytic infiltrates, which was positive for KT3 or B220, was determined as follows: At a magnification of 100, the total surface area of each infiltrate was measured by drawing a line around the infiltrate. Then the total number of pixels within this area was determined. After image thresholding, the number of diaminobenzidine positive pixels within the inflammatory infiltrate was quantified. This value was divided by the total area of the lymphocytic infiltrate (in pixels) to determine a percentage of the area of the infiltrates that stained positive for one of the lymphocytic markers. The number of infiltrates analyzed within each gland varied from 1 to 8, depending on how many infiltrates were present. If more than 8 infiltrates were present, the infiltrates to be analyzed were randomly chosen.

Microscopic analysis of levels of T lymphocytes present in SMG of NOD and MRL/lpr mice was performed by counting the numbers of $KT3^+$ cells in 2 to 4 areas of 10 mm^2 glandular tissue per mouse. Five mice per age group in both mouse strains were evaluated.

Statistical analysis

The values measured within the gland of a single mouse were averaged, after which the means of the groups were calculated. The differences between the means were evaluated by means of the Mann-Whitney two-sample two-tailed signed rank test. A p value < 0.05 was considered statistically significant.

Results

Development of focal lymphocytic infiltrates in the submandibular glands of NOD mice and MRL/lpr mice

When sections of the submandibular glands (SMG) of 5-week-old NOD mice and MRL/lpr mice were compared, focal lymphocytic infiltrates were already present in SMG of 6 of 8 MRL/lpr mice. These infiltrates were demarcated areas of lymphocytes that had not infiltrated the surrounding parenchyma. In NOD mice, focal demarcated lymphocytic infiltrates were first detected at the age of 10 weeks and were more prominent at 20 weeks, yet the areas of infiltration were not as large as those in the MRL/lpr mouse (Fig. 1, A and E). In the latter mouse strain, the large focal infiltrates had lost their area of demarcation and had started to infiltrate the surrounding parenchyma when the mice were 20 weeks of age.

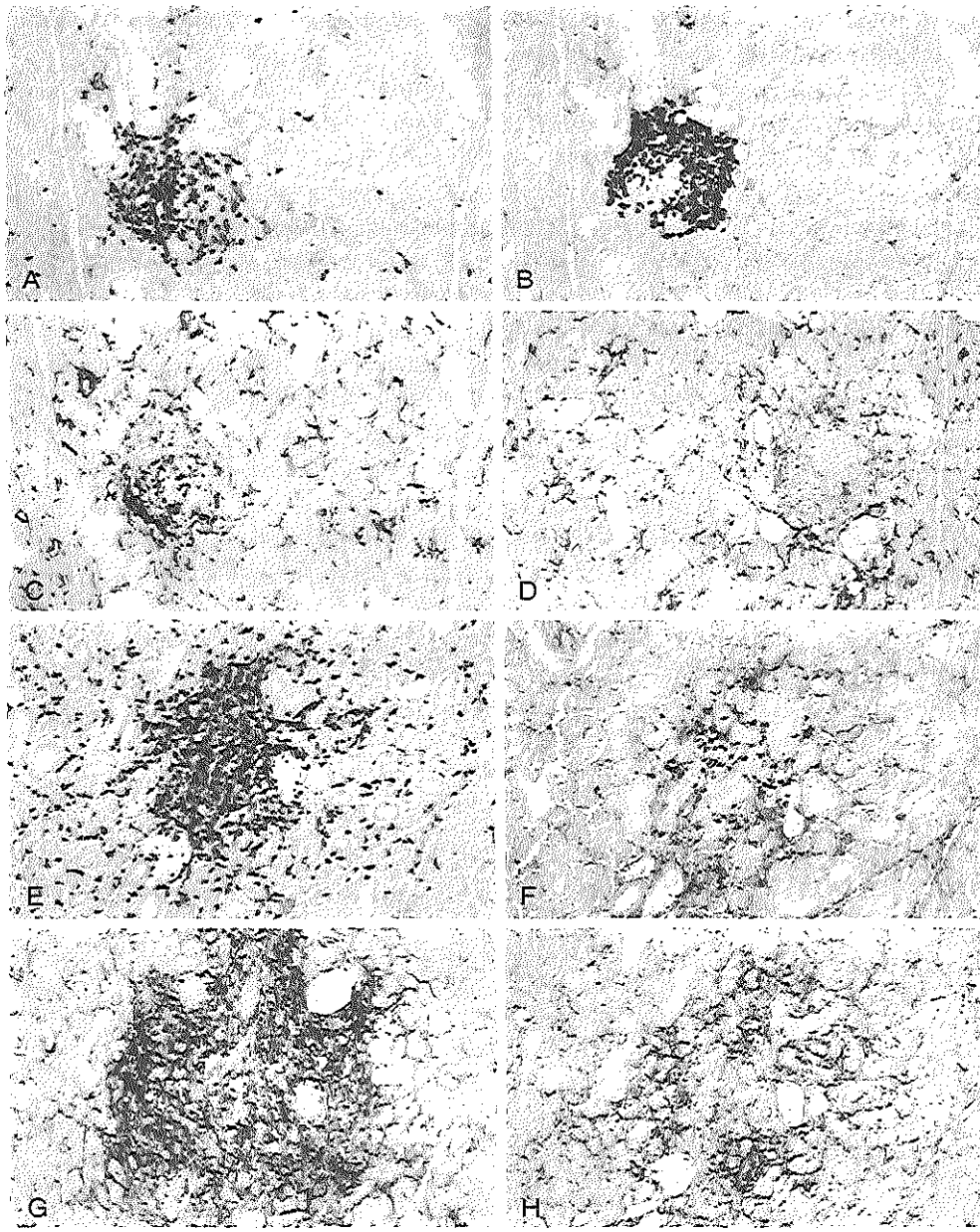

Figure 1
Immunohistochemical detection of leucocyte subsets in serial sections of lymphocytic infiltrates in a submandibular gland (SMG) of a 20-week-old nonobese diabetic (NOD) mouse and a 20-week-old MRL/lpr mouse, respectively. A to D, NOD mouse; E to H, MRL/lpr mouse. A and E, T cells identified by the MoAb KT3. B and F, B cells identified by the MoAb B220. C and G, $CD11c^+$ dendritic cells identified by the MoAb N418. D and H, $BM8^+$ macrophages (× 200).

Structure of the focal lymphocytic infiltrates in the SMG of NOD mice and MRL/lpr mice during the development of sialoadenitis

The focal lymphocytic infiltrates in the SMG of 5-week-old MRL/lpr mice showed a certain degree of structure, with separate T-cell and B-cell areas (Fig. 2, A and B). This structure had vanished in MRL/lpr mice aged 20 weeks, and distinct T-cell and B-cell areas could no longer be identified (Fig. 1, E and F), although the areas of infiltration had increased in size and lymphocytes had started to infiltrate the surrounding parenchyma. In NOD mice, the pattern of development of focal lymphocytic infiltrates was different from that in MRL/lpr mice. At 10 weeks, when focal lymphocytic infiltrates were detectable in NOD mice, there was no apparent structure; only at 20 weeks of age could clearly structured focal infiltrates be detected (Fig. 1, A and B). Areas consisting predominantly of B cells could be distinguished, while packed T cells were present in an area in which B cells were largely absent. T cells were also present as scattered cells in the B-cell area.

With regard to the presence of $CD11c^+$ DC in the focal lymphocytic infiltrates, the density of $CD11c^+$ DC was highest in the packed T-cell area of 20-week-old NOD mice (Fig. 1C). In the SMG of 20-week-old MRL/lpr mice, DC were distributed throughout the whole lymphocytic infiltrate (Fig. 1G). $BM8^+$ macrophages were detected in the SMG of NOD and

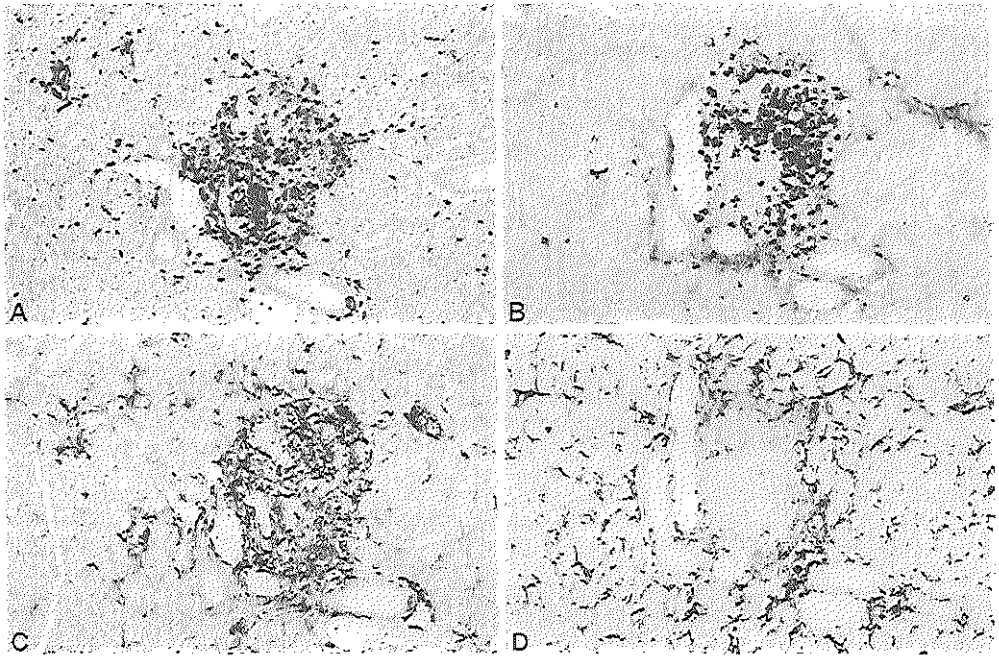

Figure 2
Immunohistochemical detection of leucocyte subsets in serial sections of a lymphocytic infiltrate showing some degree of organization in the SMG of a 5-week-old MRL/lpr mouse. A, T cells identified by the MoAb KT3. B, B cells identified by the MoAb B220. C, $CD11c^+$ dendritic cells identified by the MoAb N418. D, $BM8^+$ macrophages (× 200).

MRL/lpr mice of all ages, but the spatial relation of those macrophages towards the focal lymphocytic infiltrates differed in the two mouse strains (Figs. 1, D and H, and 2D). In the SMG of 20-week-old NOD mice, a thin rim of $BM8^+$ macrophages was present around lymphocytic infiltrates (Fig. 1D). In MRL/lpr mice, a thicker rim of $BM8^+$ macrophages was present around the lymphocytic infiltrates. In addition, these cells invaded the lymphocytic infiltrates to a certain extent (Fig. 1H).

These results show that qualitative differences exist between the focal lymphocytic infiltrates that develop in the SMG of NOD mice as compared with those in MRL/lpr mice.

Image analysis of the lymphocytic infiltrates, present within the submandibular glands of NOD mice and MRL/lpr mice

Image analysis revealed that at the onset of the development of focal lymphocytic infiltrates in the two mouse strains, no significant differences occurred in the percentages of T lymphocytes and B lymphocytes in the focal infiltrates (Table 1).

When the mice were 20 weeks old, the percentage of B cells in the fully developed focal infiltrates of the NOD mice was significantly higher than that in the MRL/lpr mice. In contrast to the percentage of B cells, the percentage of T lymphocytes in the focal lymphocytic infiltrates was significantly higher in 20-week-old MRL/lpr mice than in age-matched NOD mice. Over time, an increase in the percentage of B cells and a decrease in the percentage of T cells in the lymphocytic infiltrates of NOD mice were observed. However, this

Table 1. Composition of lymphocytic infiltrates present within submandibular glands of NOD MLR/lpr mice

Strain	Age (weeks)	B cells (%)[a]	T cells (%)[a]
NOD	10	24.7 ± 8.5	29.5 ± 4.0
NOD	15	33.3 ± 2.7	28.5 ± 6.0
NOD	20	35.2 ± 3.2	26.7 ± 3.7
MRL/lpr	5	27.0 ± 3.2	28.1 ± 5.4
MRL/lpr	10	23.5 ± 2.3#[b]	32.0 ± 2.5
MRL/lpr	15	19.7 ± 1.8* #[c]	32.1 ± 3.8
MRL/lpr	20	17.8 ± 2.3#	34.4 ± 2.1#

a Values are given as the mean ± standard deviation.

b Statistical comparison of time point with the previous time point by the Mann-Whitney two-sample two-tailed signed rank test (* $p < 0.05$).

c Statistical comparison of MRL/lpr mice with age-matched NOD mice by the Mann-Whitney two-sample two-tailed signed rank test (# $p < 0.05$).

NOD, Nonobese diabetic.

was not statistically significant. In MRL/lpr mice, a decrease in the percentage of B cells and an increase in the percentage of T cells that was not significant were observed. The decrease in the percentage of B cells in aging MRL/lpr mice was also obvious from immunohistochemical stainings (Figs. 1F and 2B).

Presence of CD11c$^+$ dendritic cells during the development of sialoadenitis in NOD mice and MRL/lpr mice

Because of the important role of DC in the activation of naive T lymphocytes, we studied the presence of DC in the SMG of NOD mice and MRL/lpr mice during the development of sialoadenitis.

In glands of 5-week-old NOD mice (before lymphocytic infiltration) and NOD-SCID mice, CD11c$^+$ cells with dendritic morphologic characteristics were scattered throughout the parenchyma of the gland. DC were scarce or absent in the SMG of 5-week-old BALB/c and C57BL/10 mice (Fig. 3). Quantification by image analysis indicated that significantly more DC were present in the SMG of 5-week-old NOD and NOD-SCID mice than in control strains (Fig. 4). To check for the earliest time point of DC infiltration in the SMG of NOD and NOD-SCID mice, we studied only a few SMG of 2-week-old mice. Hardly any DC were

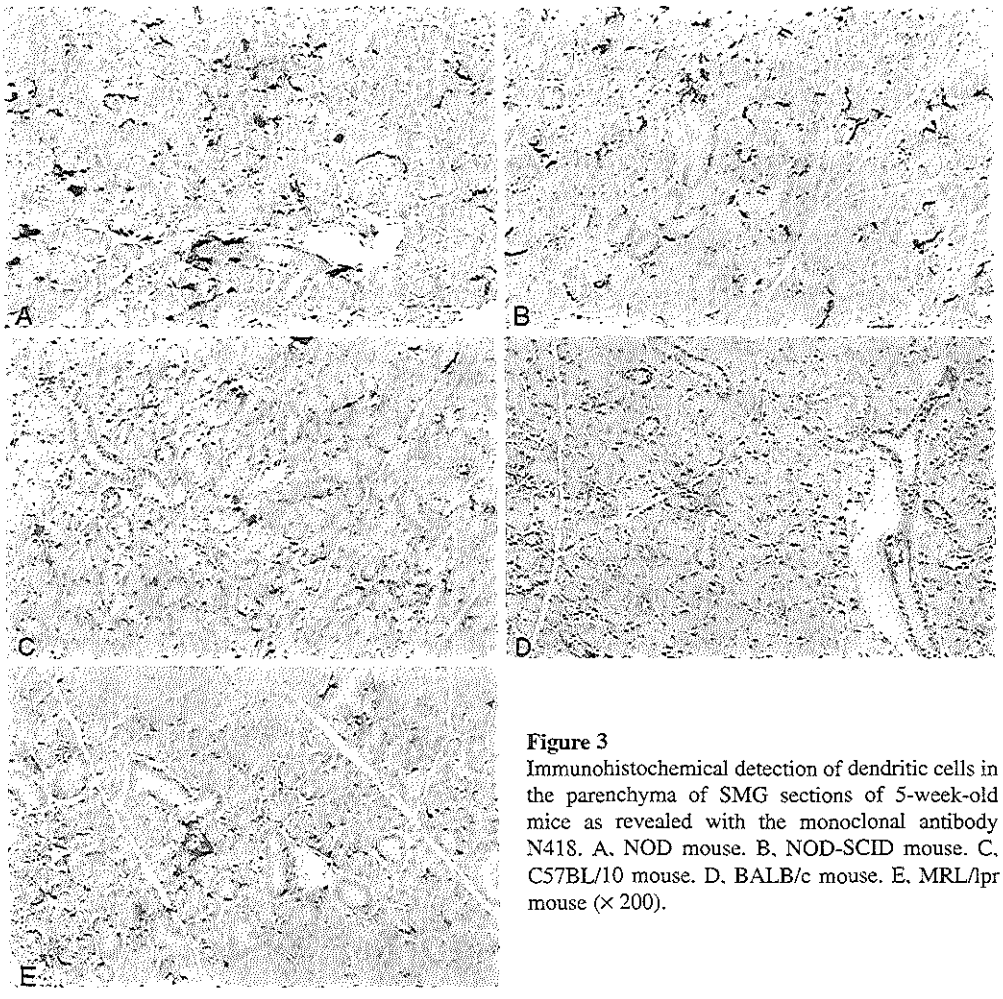

Figure 3
Immunohistochemical detection of dendritic cells in the parenchyma of SMG sections of 5-week-old mice as revealed with the monoclonal antibody N418. A, NOD mouse. B, NOD-SCID mouse. C, C57BL/10 mouse. D, BALB/c mouse. E, MRL/lpr mouse (× 200).

detected in the SMG (Fig. 5), which indicates that DC influx occurred between the age of 2 and 5 weeks.

When the influx of DC into the parenchyma of the SMG of NOD and NOD-SCID mice was studied over time, a steady increase in the number of $CD11c^+$ cells was observed until the mice were 15 weeks old, although the increase was less pronounced in NOD-SCID mice than in NOD mice (Fig. 5). In both mouse strains, a plateau was reached at the age of 15 weeks. The steady increase in DC numbers was not observed in BALB/c mice and C57BL/10 mice, which indicates that the phenomenon is not a general consequence of aging.

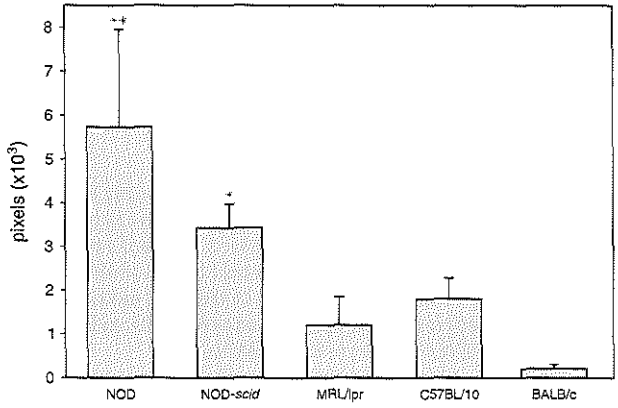

Figure 4
Dendritic cells in the SMG of 5-week-old mice. After having undergone immunohistochemical staining with MoAb N418 to identify CD11c positive dendritic cells, positively stained cells were quantified by two independent individuals who used an image analysis system. Values are expressed as means + standard deviation of 4 to 8 animals per group. Statistical analysis was performed by the Mann-Whitney two-sample two-tailed signed rank test. * Significantly different as compared with C57BL/10 mice, $p < 0.01$. ** Significantly different as compared with C57BL/10 mice, $p < 0.005$.

Few DC were present in the parenchyma of the SMG of 5-week-old MRL/lpr mice; this is comparable to the situation in C57BL/10 mice (Fig. 3, C and E). Because focal lymphocytic infiltrates were already present in this mouse strain at the age of 5 weeks, the virtual absence of DC at this age does not exclude a possible influx of DC into the SMG of MRL/lpr mice at an earlier time. To examine the relationship between DC and the first appearance of lymphocyte accumulation in the SMG of the two mouse strains, a detailed analysis on the presence of DC and T lymphocytes in the SMG of MRL/lpr and NOD mice that were 1 to 5 weeks old was performed (Fig. 6).

In the SMG of 1-week-old MRL/lpr mice, hardly any dendritic cells were present, which was comparable to the situation in 2-week-old NOD, NOD-SCID, and control mice. However, scattered T lymphocytes were already detected in the glandular tissue of those MRL/lpr mice. Accumulations of lymphocytes were present in the SMG of 3 of 5 MRL/lpr

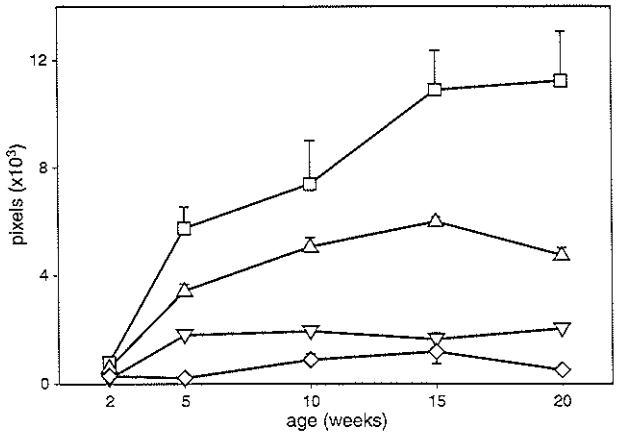

Figure 5
Dendritic cells in the SMG of different mouse strains over time. After having undergone immunohistochemical staining with MoAb N418, positively stained cells were quantified by two independent individuals who used an image analysis system. Values are expressed as means ± standard errors of the means. Values that were measured in the SMG of NOD or NOD-SCID mice 5 weeks of age or older were significantly different when compared with values that were measured in the glands of the other mouse strains (p, $0.001 < p < 0.05$). However, there was no significant difference between 10-week-old NOD and NOD-SCID mice. = NOD mice; Δ = NOD-SCID mice; ∇ = C57BL/10 mice; ◊ = BALB/c mice.

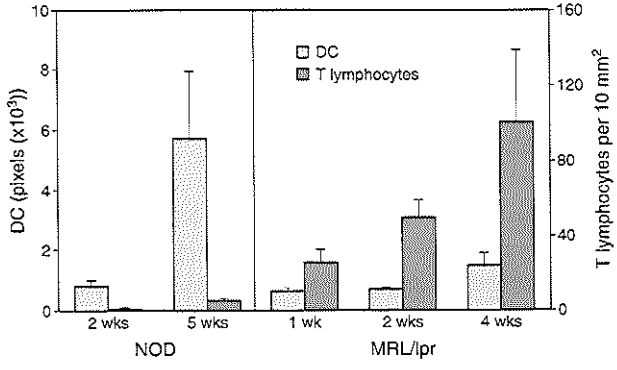

Figure 6
Analysis of dendritic cells and T lymphocytes in the SMG of NOD and MRL/lpr mice that were 1 to 5 weeks old. Positively stained dendritic cells were quantified by two independent individuals who used an image analysis system (left y-axis). The numbers of positively stained T lymphocytes were counted per 10 mm^2 glandular tissue by two independent individuals (right y-axis). Values are expressed as means + standard deviation of 5 animals per group.

mice that were 2 weeks old, and the number of scattered T lymphocytes had increased. In the surrounding parenchyma, very few DC were present. Small lymphocytic infiltrates were detected in the SMG of 4-week-old MRL/lpr mice, while a slight increase in the number of dendritic cells was observed. In the SMG of MRL/lpr mice 6 to 20 weeks old, the increase in the number of DC in the parenchymatous tissue of the gland was modest over time (results not shown). Still, numbers of DC were clearly lower in 20-week-old MRL/lpr mice than in NOD mice of the same age.

Discussion

This study shows two distinct patterns of autoimmune sialoadenitis development in the NOD mouse and the MRL/lpr mouse, which are two mouse models for Sjögren's syndrome. In NOD mice, the presence of focal lymphocytic infiltrates (first not structured and later with T-cell and B-cell areas) was first observed when the mice were 10 weeks old and was preceded by an influx of DC that began between the age of 2 and 5 weeks. This influx of DC was in both independent of and dependent on the presence of lymphocytes, because such an influx was also observed in NOD-SCID mice, but to a lesser degree. NOD-SCID mice lack functional T lymphocytes and B lymphocytes.

In the SMG of MRL/lpr mice, focal lymphocytic infiltrates were already present at the age of 5 weeks, while first signs of lymphocytic accumulation were already observed at the age of 2 weeks. The virtual absence of DC in the SMG of 1-week-old MRL/lpr mice shows that the development of focal lymphocytic infiltrates in this mouse strain is not preceded by an influx of DC in the parenchyma of the gland. Because slightly increased numbers of DC in the parenchyma of the SMG were observed in mice ≥ 4 weeks old (ages at which lymphocytic infiltrates had started to develop), it can be concluded that DC in MRL/lpr mice ≥ 4 weeks old are due to the presence of lymphocytic infiltrates.

These two distinct patterns of DC influx and focal infiltrate development in the two mouse models suggest two distinct patterns of disease pathogenesis. With regard to the early DC accumulation in the parenchyma of the SMG of NOD mice before any noteworthy lymphocytic infiltration, it is likely that DC pick up autoantigens in the SMG of the NOD mouse and travel to the draining lymph node. In the lymph node, DC activate naive T cells to initiate the autoimmune process, which later leads to the focal accumulation of T cells in the SMG. The traffic of DC through tissues is tightly regulated by chemokine receptor expression on the cells. During their maturation from monocytes, DC express various combinations of chemokine receptors that enable monocytes and DC, depending on their maturational stage, to respond to different chemokines (22, 23). The migration of epidermal Langerhans cells and dermal DC can be enhanced by the cytokines IL-1β and TNF-α (24). Recently, chemokine expression in the minor salivary glands (MSG) of patients with Sjögren's syndrome has been described, and ductal epithelial cells were identified as the predominant source of chemokines in the MSG (25). An increased expression of the cytokines IL-1α, IL-

1β, TNF-α, and IL-6 by salivary gland epithelial cells of Sjögren's syndrome patients when compared with controls has been observed (26-28). These studies illustrate the capability of epithelial cells of the salivary glands to express cytokines that may attract DC. Such enhanced chemokine-cytokine expression might be linked to a metabolic abnormality or disturbed proliferation of the salivary gland epithelial cells of the NOD mouse; note that changes in the salivary protein composition in aging NOD mice have been observed (6, 7). Early metabolic and/or growth abnormalities that precede the DC influx have also been noted in the thyroid gland of the biobreeding diabetes-prone (BB-DP) rat, in which autoimmune thyroiditis develops after the influx of DC (29).

In MRL/lpr mice, the focal sialoadenitis started in the virtual absence of DC in the parenchyma at a much earlier age than in NOD mice. This suggests that the SMG epithelial cells are not the initial driving force behind the autoimmune process. It is possible that in MRL/lpr mice, T cells and B cells, sensitized to an autoantigen that is not primarily expressed in the salivary glands, are already present from a very early age. This antigen might be of nuclear origin, like double-stranded DNA. The production of antibodies towards such an autoantigen can lead to the formation of immune complexes and to the deposition of these complexes in the small vessels, which is followed by vasculitis. Sensitization of lymphocytes is in that case not primarily mediated by APC coming from the salivary gland but instead takes place elsewhere in the body.

Another important difference between sialoadenitis in NOD and MRL/lpr mice was the architecture of the focal lymphocytic infiltrates. In both mouse models, focal lymphocytic infiltrates developed during the course of the disease. The lymphocytic infiltration occurred in NOD mice 10 weeks of age and older and gradually gained structure with T-cell and B-cell areas. In 5-week-old MRL/lpr mice, structured focal infiltrates were already present, but that structure was lost over time.

What is the function of the organized focal lymphocytic infiltrates that develop in the SMG of both strains of mice? Similarly organized structures have been observed in other animal models of organ-specific autoimmune disease, e.g. the thyroid gland of the BB rat (30) and the pancreas of the NOD mouse (31). These organized focal infiltrates, some aspects of which resemble normal lymph node and gut-bronchus associated lymphoid tissue, can be places of peripheral T-cell and B-cell sensitization to serve local generation of autoreactive T cells and the production of autoantibodies. In the BB rat, a correlation was observed between the development of intrathyroidal focal lymphoid cell infiltrates and the incidence of anticolloid antibodies, which suggests that within such organized lymphoid structures, thyroid autoantibodies are produced (30). A continued presence or release of antigen from the salivary gland is a probable driving force behind the development of the SMG-associated lymphoid tissue: *De novo* formation of organized lymphoid structures after repeated challenge with an organ-specific antigen was recently observed in a transgenic diabetic mouse model. Transgenic mice expressing the lymphocytic choriomeningitis virus glycoprotein under the control of the rat insulin promotor were immunized with DC that expressed an immunodominant cytotoxic T-lymphocyte epitope of the viral glycoprotein. Pancreatic islet

associated organized lymphoid structures developed in these mice in addition to destructive autoimmune diabetes (32). In the MSG of two patients with Sjögren's syndrome, organized lymphocytic infiltrates have recently been described (33). In an MSG of one of these patients, fully developed germinal centers surrounded by large numbers of plasma cells were detected. It was suggested that the continued presence of self-antigen in the salivary gland was responsible for the development of these highly organized structures. Anti-Ro/SSA and anti-La/SSB autoantibody-producing cells have been detected in the MSG of patients with Sjögren's syndrome (34).

When the composition of the focal inflammatory infiltrates was studied, a higher percentage of B cells was observed in 15- or 20-week-old NOD mice than in 10-week-old NOD mice. This age-related increase was not observed in MRL/lpr mice. We believe that this is due to a different pattern of cytokine production by the cells of the inflammatory infiltrates. The high number of T cells and of $BM8^+$ macrophages in the focal infiltrates present in the SMG of MRL/lpr mice suggests the overproduction of proinflammatory Th1 cytokines that are important in the stimulation of cellular immune reactions. Far fewer $BM8^+$ macrophages and a predominance of B cells were observed in the SMG of NOD mice when compared with the SMG of MRL/lpr mice. This picture would fit more into a Th2-type reaction.

Despite our histological observation that the glandular tissue in the SMG of 20-week-old MRL/lpr mice was more damaged, a decreased stimulated saliva production in this mouse strain has not been reported. This is in contrast to what has been described for the saliva production in NOD mice (6, 7), in which we did not find signs of parenchymal decay. The lack of glandular hypofunction in the MRL/lpr mouse in spite of signs of destruction of glandular tissue is explained by the fact that in general, a major part of the glandular tissue must be destroyed before an insufficient production of glandular product results. In NOD mice, salivary gland hypofunction is probably mediated by blocking antibodies, because salivary gland hypofunction could be transferred to young NOD mice by intraperitoneal injection of serum from old NOD mice (35). This emphasizes the importance of Th2 type reactivity in this model of Sjögren's disease. Moreover, the intrinsic abnormalities of the NOD salivary gland epithelial cells may play a role here.

If our data are extrapolated from mice to humans, different pathogenetic mechanisms for sialoadenitis (Sjögren's syndrome) exist. NOD-like sialoadenitis would follow the pattern of development of the known other organ-specific autoimmune diseases such as type 1 diabetes and thyroiditis. This primary form of Sjögren's syndrome is characterized by an antigen of salivary gland origin and perhaps a histopathologic picture akin to that of the NOD mouse: Structured focal infiltrates, a predominance of B cells within the infiltrates, and a strong parenchymal infiltration of DC. MRL/lpr-like sialoadenitis would follow a different pattern of development. This form would be secondary and would be associated with systemic autoimmune disorders and perhaps a histopathologic picture akin to that of the MRL/lpr mouse: Unstructured focal infiltrates, a predominance of T cells within the infiltrates, and a parenchymal infiltration with predominantly scavenger macrophages.

Our data in the animal models indicate that a detailed analysis of the composition of

the infiltrates in the MSG of patients with Sjögren's syndrome (B cells, T cells, DC, macrophages) is worthwhile for a differential diagnosis. This subject will form the core of our next report.

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Chapter 3

PROFESSIONAL ANTIGEN PRESENTING CELLS IN MINOR SALIVARY GLANDS IN SJÖGREN'S SYNDROME: POTENTIAL CONTRIBUTION TO THE HISTOPATHOLOGICAL DIAGNOSIS?

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Abstract

Sjögren's syndrome is an autoimmune disease in which lymphocytic infiltrates develop in the salivary and lacrimal glands. We have shown that dendritic cells (DC) infiltrate the submandibular gland of the nonobese diabetic (NOD) mouse, a mouse model for Sjögren's syndrome, before lymphocytic infiltration, suggesting that these antigen presenting cells (APC) may play a role in the initiation of Sjögren's syndrome. In later stages, DC and macrophages also form an important part of the infiltrate of the NOD sialoadenitis. To find out if DC and macrophages form part of the infiltrate in Sjögren's syndrome as well, and to determine whether they may be usefull in the histopathological diagnosis of Sjögren's syndrome, we studied their presence in minor salivary glands (MSG) of patients with Sjögren's syndrome and patients with focal lymphocytic sialoadenitis (FLS), but without clinical or serological criteria of Sjögren's syndrome. Immunohistochemistry was applied, followed by semiquantitative analysis.

DC and macrophages were present in all MSG; however, there were clear differences in marker expression between Sjögren's syndrome and FLS, on the one hand, and control tissue, on the other hand. CD1a$^+$ DC and RFD9$^+$ macrophages were mainly observed in MSG in which a focal lymphocytic infiltrate was present. In fact, the diffuse presence of single CD1a$^+$ DC and RFD9$^+$ macrophages correlated closely with the presence of a focal lymphocytic infiltrate in the MSG. This indicates that these cells could be of help during the evaluation of a MSG. Because the detection of APC is technically less cumbersome than a focal score, this parameter may perhaps replace the focal score in the histopathological diagnosis of Sjögren's syndrome. This study therefore prompts further investigation focusing on the presence of CD1a$^+$ and RFD9$^+$ cells in the MSG of a large cohort of patients.

Introduction

Sjögren's syndrome is a chronic autoimmune disease characterized by the presence of lymphocytic infiltrates in the salivary and lacrimal glands. The presence of these infiltrates is accompanied by decreased saliva and tear production. This is ultimately manifested by xerostomia and keratoconjunctivitis sicca (KCS) (1, 2). Systemic manifestations, like arthritis, vasculitis, and serum autoantibodies directed to the ribonuclear proteins SS-A and SS-B, may be present as well (3). A combination of immunologic, genetic, hormonal, and viral factors have been implicated in the pathogenesis of Sjögren's syndrome, but the initiating event of the autoimmune reaction is still not known (3-5).

Initiation of an (auto)immune response starts with the presentation of an antigen to antigen-specific CD4$^+$ T cells by antigen presenting cells (APC). APC include dendritic cells (DC), macrophages, and B cells (professional APC), but also nonprofessional APC, like epithelial cells. So far, with regard to the presence of leucocyte subsets in minor salivary

glands (MSG) of patients with Sjögren's syndrome, attention has primarily been paid to the lymphocytic composition of the focal infiltrates (6, 7). Antigen-presenting cells have received little attention.

We have recently shown that, before the development of lymphocytic infiltrates, DC accumulate in the submandibular glands of nonobese diabetic (NOD) mice, a mouse model for Sjögren's syndrome (8, 9). In the MRL/lpr mouse, another mouse model for Sjögren's syndrome (10), hardly any DC were present before the onset of sialoadenitis (11). This suggests that DC play an important role in the initiation of sialoadenitis in NOD mice, whereas in MRL/lpr mice their role may be limited. In addition to the presence of DC in the early phase of sialoadenitis in NOD mice, DC as well as macrophages formed an important component of the focal infiltrates in a later phase of the autoimmune process.

The presence of DC and macrophages in the submandibular glands of NOD mice and the potential role of these cells in the initiation and perpetuation of the autoimmune reaction prompted us to study the presence of these cell types in the MSG of patients with Sjögren's syndrome.

The MSG biopsy has an important role in establishing the diagnosis Sjögren's syndrome in all sets of criteria that are being used. However, evaluation of the focus score is a tedious procedure and requires the examination of more than one section by an experienced pathologist. Because histopathological involvement may be patchy, focal lymphocytic infiltrates of more than 50 mononuclear cells may be missed if only one section is used to examine the presence of a lymphocytic infiltrate. Furthermore, it has been estimated that the false positive rate of the minor salivary gland biopsy may be as high as 20%, whereas inadequate scoring may be as high as 19% (12).

We therefore examined in a limited number of patients whether the presence of DC and macrophages in the MSG can be used as a scoring parameter for the histopathological diagnosis of Sjögren's syndrome, and whether the presence of these APC correlates with the presence of focal lymphocytic infiltrates. We not only used tissue from well-established Sjögren's syndrome patients, fulfilling the European criteria (ie, focal lymphocytic sialoadenitis along with symptoms and positive tests for dry eyes and dry mouth, and serum antibodies directed towards SS-A or SS-B), but also from patients with focal lymphocytic sialoadenitis only, without further clinical or serological criteria of Sjögren's syndrome (FLS). Patients without focal sialoadenitis and oral complaints, but with the clinical eye symptoms of Sjögren's syndrome, ie, keratoconjunctivitis sicca (KCS), were used as a negative disease control. Patients with complaints of Sjögren's syndrome, but lacking any clinical or pathological criteria for the disease, were also biopsied for diagnostic purpose. The tissues of these cases were used as negative controls.

Briefly, the overall aim of this study was to investigate the presence of dendritic cells and macrophages in MSG and their potential contribution to the pathological practice.

Materials and methods

Patients and controls

Lipbiopsies were obtained from various groups of patients. (a) Samples were taken from 15 patients (14 women and 1 man; ages 32 to 79 years) with Sjögren's syndrome according to the European Community Criteria (31). In the serum of five of these patients, antinuclear antibodies or anti-SS-A or anti-SS-B antibodies had been detected. All 15 patients also fulfilled the newly proposed European criteria for Sjögren's syndrome that require a positive focus score or antibodies to SS-A or SS-B in each patient (32). (b) Samples were obtained from six patients (all women, ages 29 to 58 years) with focal lymphocytic sialoadenitis (FLS) only. Patients with FLS were characterized by the presence of focal lymphocytic infiltrates with a focus score equal to or greater than 1 in their MSG, but without keratoconjunctivitis sicca (KCS). Thus, these patients did not fulfill the clinical criteria to be diagnosed as having Sjögren's syndrome. (c) Samples were also obtained from eight patients (7 women, 1 man; ages 31 to 56 years) with KCS, without a completely developed Sjögren's syndrome. Patients with KCS were diagnosed on the basis of serious complaints of dry eyes, a positive Van Bijsterveld score, and an abnormal Schirmer test or break-up time. The outcome of the Schirmer test was considered abnormal if less than or equal to 5 mm. The break-up time was considered abnormal if the outcome was lower than 11 seconds. (d) As control tissue, lip biopsies from 17 patients (15 women, 2 men; ages 34 to 76 yrs) were studied. These patients suffered from sicca complaints, but were negative in objective diagnostic tests for ocular and oral involvement. Neither antinuclear antibodies nor anti-SS-A or anti-SS-B antibodies were detected in the serum of any of the control patients. Lip biopsies had been performed in all patients for routine diagnosis. Biopsies were either fixed in 10% buffered formalin or snap-frozen within 30 minutes in Tissue-tek embedding medium (Sakura Finetek, Torrance, California), using liquid nitrogen. Biopsies that were snap-frozen were stored at –80°C and used for research purposes.

Immunohistochemistry

Frozen sections (6 μm) from each biopsy were placed on poly-L-lysine-coated slides (Sigma, Diagnostics, St Louis, Missouri) and fixed in aceton for 10 minutes at room temperature. Afterwards, the slides were rinsed in PBS (pH 7.8) for 5 minutes and incubated with 1% bovine serum albumine in PBS for 10 minutes. Subsequently the slides were incubated with 10% normal rabbit serum (Dakopatts, Glostrup, Denmark) in PBS for 10 minutes, after which one of the monoclonal antibodies (MoAb) was applied (Table 1). L25 is a MoAb directed against B cells and dendritic cells (13) and was a generous gift of Dr. T. Takami (Department of Pathology, School of Medicine, Gifu University, Gifu, Japan). RFD7 and RFD9 are MoAb directed against macrophage subsets (18), and these were kindly provided by Dr. L.W. Poulter (Department of Immunology, Royal Free Hospital, London, United Kingdom). The optimal dilutions were determined by titration. The slides were incubated

with the primary antibody of interest for 60 minutes, rinsed in PBS for 10 minutes, and incubated for 30 minutes with rabbit antimouse (RαM) immunoglobulin antiserum (175 μg/ml) (Dako A/S, Glostrup, Denmark).

Table 1. Monoclonal antibodies used in this study.

Antibody	Main specificity	Source
RFD7	Macrophages	Dr. L.W. Poulter, London, UK
RFD9	Macrophages	Dr. L.W. Poulter, London, UK
L25	B cells, dendritic cells	Dr. T. Takami, Gifu, Japan
OKT6	CD1a	Ortho Diagnostics, Raritan, NJ
HB15a	CD83	Immunotech S.A., Marseille, France

Subsequently, the sections were rinsed in PBS, incubated with alkaline phosphatase anti-alkaline phophatase (5 μg/ml) (DAKO A/S) for 30 minutes, rinsed in TRIS buffer (pH 8.0), and incubated for 30 minutes with New Fuchsin substrate (Chroma, Stuttgart, Germany) which stained positive cells red. Finally, the slides were rinsed with water, counterstained with Mayer's hematoxylin (Merck diagnostica, Darmstadt, Germany), and mounted in Kaiser's glycerol gelatin (Merck diagnostica). Control staining was performed by substitution of the primary antibody with PBS and by incubation with an irrelevant monoclonal antibody of the same isotype and concentration. Stained sections were evaluated blindly by three independent persons (SvB, AW-W, CvH-M), using a semiquantitative 0 to 3 scale (grade 0, no positive cells; grade 1, 1-5 positive cells per 0.625 mm^2; grade 2, 6-30 positive cells per 0.625 mm^2; grade 3, 30-100 positive cells per 0.625 mm^2). A distinction was made between cells staining positive located within a focal infiltrate and cells staining positive located in glandular tissue. In sections of MSG of patients with Sjögren's syndrome or FLS that were used to study one of the markers of interest, a lymphocytic infiltrate was not always present. In these cases however, the presence of a focal lymphocytic infiltrate was detected in other sections of these MSG.

Results

Dendritic cells in minor salivary glands

Three separate antibodies were used to study the presence of DC in MSG. L25 reacts with interdigitating cells in the thymus-dependent areas of peripheral lymphoid organs, and with B cells (13). $CD83^+$ cells represent mature DC (14, 15), whereas $CD1a^+$ DC represent DC positive for a molecule that plays a role in the presentation of lipid and glycolipid antigens (16, 17). Whereas $L25^+$ and $CD83^+$ DC were found in virtually all MSG, $CD1a^+$ DC were specific for MSG in which a focal lymphocytic infiltrate was present.

$L25^+$ cells were present in all studied MSG, regardless of the presence of a lymphocytic infiltrate within the MSG (Figs. 1 and 2, A and B). If there were focal lymphocytic infiltrates, the highest numbers of $L25^+$ cells were normally observed within these infiltrates. To discriminate between DC and B cells, the presence of $CD19^+$ cells (B cells) within the MSG

was also examined. Based on these stainings, we concluded that part, but not all, of the $L25^+$ cells within the lymphocytic infiltrates were indeed B cells. In the parenchyma of the MSG, $L25^+$ cells were mainly DC, which was also suggested by the dendritic morphology of the cells.

$CD83^+$ cells were present in the MSG of patients with Sjögren's syndrome or FLS, as well as in controls (Fig. 1). In MSG with a lymphocytic infiltrate, CD83 expression was found within parenchymatous tissue as well as in the lymphocytic infiltrate (Fig. 2D).

$CD1a^+$ DC were found in the MSG of all patients with Sjögren's syndrome and of the majority of patients with FLS (Fig. 1). Such cells were only present in 20% of the control

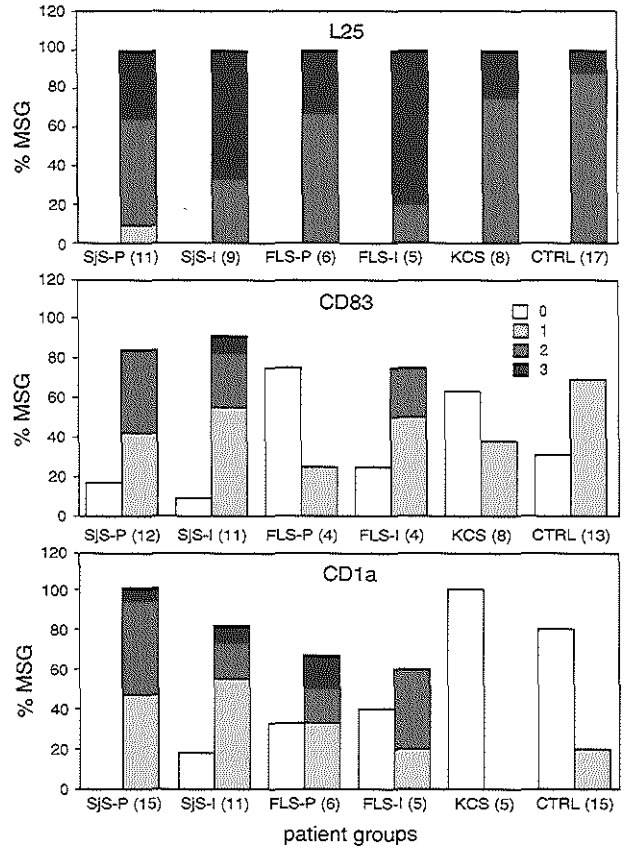

Figure 1
Semiquantitative analysis of dendritic cell subsets in minor salivary glands (MSG) of patients with Sjögren's syndrome (SjS), focal lymphocytic sialoadenitis (FLS), and keratoconjunctivitis sicca (KCS), as well as in those of control subjects (CTRL). In MSG of patients with SjS and FLS, a distinction was made between positively staining cells located in the parenchyma (P) and those in the lymphocytic infiltrate (I). The number of MSG studied is shown in parentheses for each condition. The following grading system was used: 0, no positive cells; 1, 1-5 positive cells per 0.625 mm^2; 2, 6-30 positive cells per 0.625 mm^2; 3: 30-100 positive cells per 0.625 mm^2. See Table 1 for the specificity of the monoclonal antibodies.

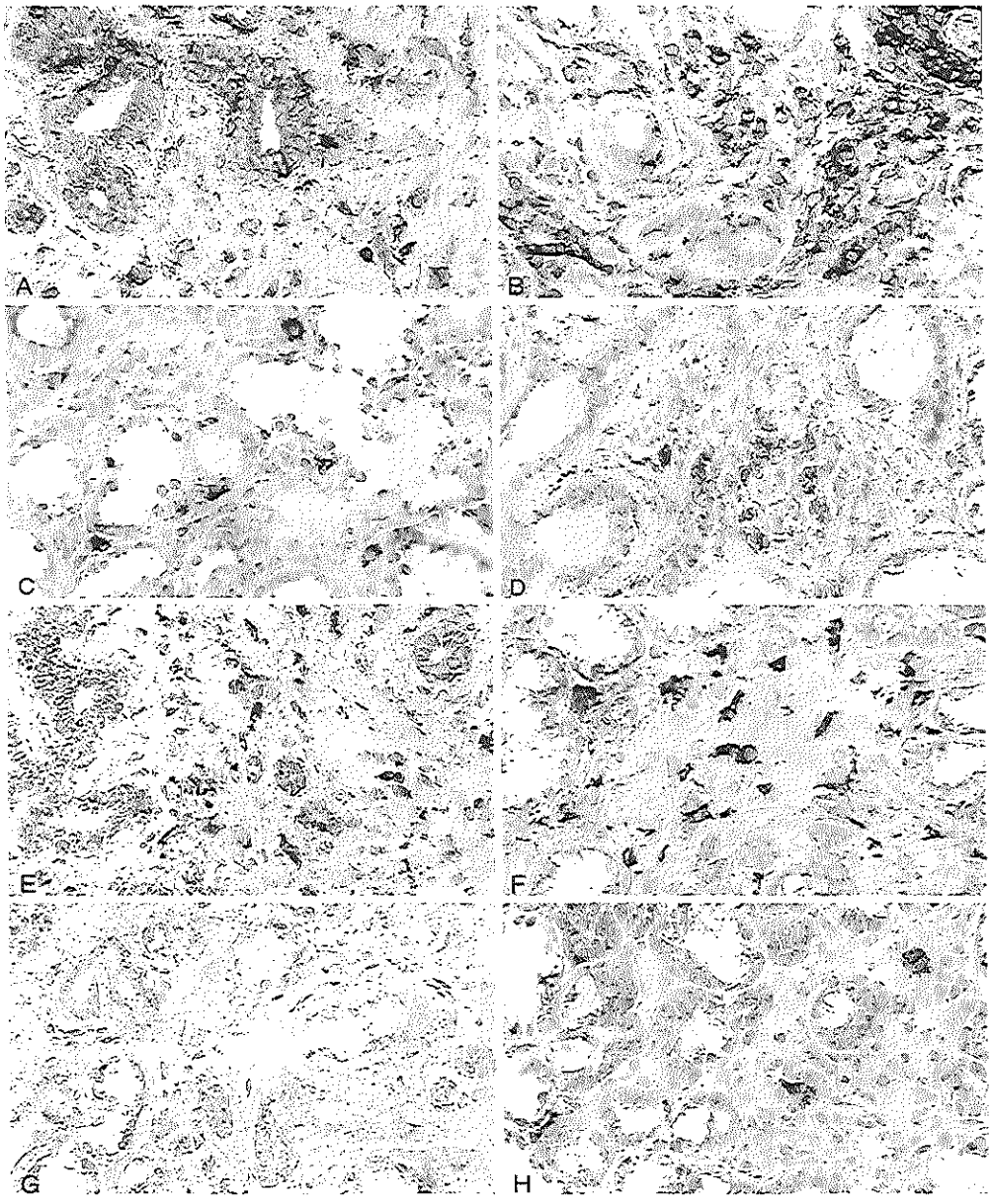

Figure 2
Immunohistochemical detection of macrophages and dendritic cell subsets in MSG. A and B, $L25^+$ dendritic cells present in a MSG of a control (A), and in that of a patient with FLS (B). C and D, $CD1a^+$ (C) and $CD83^+$ (D) dendritic cells present in the parenchyma of a MSG of a patient with Sjögren's syndrome. E and F, $RFD7^+$ macrophages, present in a MSG of a control subject (E), and in that of a patient with FLS (F). G and H, $RFD9^+$ macrophages present in a MSG of a control subject (G), and in that of a patient with Sjögren' syndrome (H). Magnification, × 400.

patients, whereas they were absent in MSG of patients with KCS. In MSG in which a lymphocytic infiltrate was present, CD1a$^+$ DC occurred both in the parenchyma of the gland (diffusely distributed) (Fig. 2C) and in the lymphocytic infiltrates.

Macrophage subsets in minor salivary glands

The MSG of patients with Sjögren's syndrome, FLS, KCS, and controls were studied for the presence of RFD7$^+$, mature tissue macrophages (18), and RFD9$^+$ macrophages, representing epithelioid cells (19). RFD7$^+$ macrophages were present in all MSG, regardless of the presence of a focal lymphocytic infiltrate (Figs. 2, E and F, and 3). In MSG in which a lymphocytic infiltrate was present, RFD7$^+$ macrophages were detected both within the infiltrate and scattered throughout the glandular parenchyma.

RFD9$^+$ cells were not so widely distributed. These macrophages were present in the parenchyma of the majority of patients with Sjögren's syndrome and FLS (71% and 80%, respectively), whereas they were largely absent in the control MSG (Figs. 2, G and H, and 3). In MSG with a lymphocytic infiltrate, RFD9$^+$ cells were present both within the lymphocytic infiltrate and in the parenchyma.

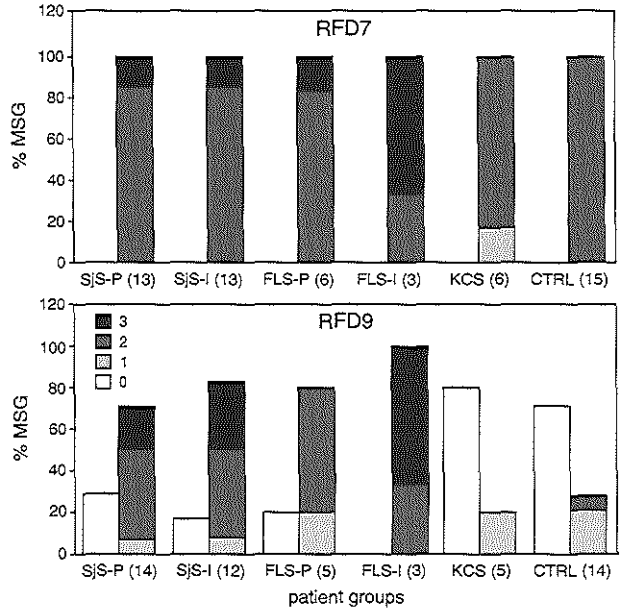

Figure 3
Semiquantitative analysis of macrophages subsets in MSG of patients with SjS, FLS, and KCS, as well as in those of control subjects. In MSG of patients with SjS and FLS, a distinction was made between positively staining cells located in the parenchyma (P) and those in the lymphocytic infiltrate (I). For details, see the legend for Figure 1 and Table 1.

Correlation between the presence of CD1a$^+$ dendritic cells and RFD9$^+$ macrophages and the presence of lymphocytic infiltrates in MSG

CD1a$^+$ DC and RFD9$^+$ macrophages were mainly present diffusely distributed in MSG in which also a lymphocytic infiltrate was also present. Because these cells were also detected in cases where the lymphocytic infiltrate was missed in the section used for the immunohistochemical staining, we examined whether the presence of these cell types in the parenchyma of the MSG correlates with the presence of a lymphocytic infiltrate.

Thirty-four MSG in which both markers were examined were divided into two groups: one group in which a positive focus score had been reported by the pathologist or was detected in this investigation in any section used for the immunohistochemical stainings, and another group in which lymphocytic infiltrates had never been observed, neither by the pathologist nor by us. The presence of CD1a$^+$ and RFD9$^+$ cells in both patient groups is listed in Table 2.

Table 2. Correlation between presence of focal lymphocytic infiltrates and presence of CD1a$^+$ and/ or RFD9$^+$ cells in parenchyma of minor salivary gland biopsies.

Focus score	Number of MSG *	CD1a$^+$ RFD9$^+$	CD1a$^+$ RFD9$^-$	CD1a$^-$ RFD9$^+$	CD1a$^-$ RFD9$^-$
Positive	19 (13)	14/19	4/19	0/19	1/19
Negative	15 (15)	0/15	2/15	5/15	8/15

* In parentheses, the number of MSG included in this part of the study that were scored positive or negative by the pathologist.

The first observation that follows from Table 2 is that, in 6 of 19 MSG (32%) in which we detected in our observations a positive focus score in any of the sections used for the immunohistochemical stainings, a negative focus score had been reported to the clinician by the pathologist. This underscores the notion that false negative scoring of the MSG biopsy is indeed a problem encountered by the pathologist when evaluating a MSG in routine procedures.

In 18 of 19 MSG (95%) in which a positive focus score was detected, CD1a$^+$ DC were present. In 14 of these MSG, RFD9$^+$ macrophages were also found. In total, CD1a$^+$ cells were found in 20 of 34 examined MSG, in 90% of which (18 of 20) a lymphocytic infiltrate was present, showing that the presence of CD1a$^+$ DC correlates significantly with the presence of a lymphocytic infiltrate. RFD9$^+$ macrophages, though mainly present in MSG in which a lymphocytic infiltrate was present, were also found in 33% (5 of 15) of MSG with a negative focus score.

Discussion

In this study the presence of DC and macrophages was examined in the MSG of patients with Sjögren's syndrome, related diseases, and controls. We found the presence of CD1a$^+$ DC (and to a lesser extent that of RFD9$^+$ macrophages) to correlate with the presence

of focal adenitis and hence to be rather specific for the histopathology accompanying Sjögren's syndrome. Because DC and macrophages are present, scattered throughout the glandular parenchyma, the detection of these cells in routine practice is likely to be easier for the pathologist than determination of the focal score, which needs more sections and levels of the gland to be evaluated. In our study, the percentage of MSG scored false negative in routine H&E sections was shown to be as high as 32% when compared with the percentage of positive focal scores in sections used for immunohistochemistry. In over 80% of these latter MSG (5 out of 6), CD1a$^+$ DC were present.

Our study thus urges for a systematic, prospective, and well-controlled investigation of the presence of CD1a$^+$ and RFD9$^+$ cells in a large cohort of patients to see if these markers can indeed be helpful in the histopathological diagnosis of Sjögren's syndrome and thus replace the tedious focal scoring. To further illustrate the necessity for critically viewing the outcome of the focal scoring, it has recently been described that smoking habits of patients might invalidate the use of the focus score in a MSG, because smoking lowers the focus score by reducing the accumulation of lymphocytes in the salivary glands (20). Reduction of either the number or the size of lymphocytic infiltrates increases the problem of false negative MSG. Whether it affects infiltration by CD1a$^+$ DC and RFD9$^+$ macrophages needs further investigation.

Our study also shows that DC and macrophages are normal components of the MSG, albeit the cells are then almost exclusively CD1a and RFD9 negative (they are L25 and CD83 or RFD7 positive). RFD7$^+$ macrophages, detected in all MSG irrespective of the presence of a lymphocytic infiltrate, probably represent a resident macrophage population that is able to eliminate debris particles and microorganisms that invade the salivary gland (classical histiocytes). We found similar macrophages in submandibular glands of the NOD mouse, the MRL/lpr mouse, and control strains (unpublished observations).

DC are the most potent APC and are capable of activating naïve T lymphocytes (21). A role for these cells, early in the process leading to the development of sialoadenitis can be envisaged. It can be imagined that L25$^+$ DC, possibly activated by events that may only occur in MSG of patients with Sjögren's syndrome or FLS, take up an autoantigen from the glandular tissue, travel to the draining lymph node, and activate antigen-specific T lymphocytes. Activation and maturation of DC can be achieved by antigen uptake and processing or by exposure of DC to inflammatory agents (22, 23).

The antigen CD1a, recognized by the antibody OKT6, is normally expressed on Langerhans cells within the epidermis of the skin (24). In this study we described the presence of CD1a$^+$ cells in MSG in which a lymphocytic infiltrate has developed. Other inflammatory conditions in which CD1a$^+$ cells have been detected include the lesions of patients with sarcoidosis. Perivascular areas of skin lesions, as well as granulomas within the lymph nodes and lungs of patients, were found to contain CD1a$^+$ cells (25). These results combined with ours suggest that the presence of CD1a$^+$ cells in the periphery may be due to a chronic inflammatory environment. With regard to up-regulation of CD1a *in vitro*, maturation of DC from CD14$^+$ progenitors, induced by granulocyte-macrophage colony stimulating factor

(GM-CSF) and tumor necrosis factor-α (TNF-α), is accompanied by increased expression of CD1a (26). Interestingly, TNF-α mRNA and protein expression have been detected in the MSG of patients with Sjögren's syndrome, as well as in those of healthy volunteers. The highest levels were detected in patients with Sjögren's syndrome (27-29). The expression of CD1a on DC in MSG in which a lymphocytic infiltrate has developed may be the result of the production of the proinflammatory cytokine TNF-α by infiltrating cells. Alternatively, it may have occurred prior to the development of lymphocytic infiltrates, perhaps because of increased expression of TNF-α by epithelial cells, and may be an early event in the development of sialoadenitis.

The presence of RFD9$^+$ macrophages, mainly in MSG with a lymphocytic infiltrate, is probably a reflection of the chronic inflammatory process characteristic of Sjögren's syndrome. RFD9$^+$ macrophages have also been described in granulomas of patients with sarcoidosis, and although their precise role is unknown, it has been suggested that clustered RFD9$^+$ macrophages are involved in antigen processing and may contribute to the persistence of chronic inflammatory (autoimmune) reactions (30). Whether RFD9$^+$ macrophages are recruited to the MSG because of cytokine production by cells of the focal infiltrate or whether they mature from a precursor subset is not known.

In conclusion, in this study we have shown that professional APC are present in MSG, and a role for these cells in the development of autoimmune sialoadenitis can be envisaged. Furthermore, the presence of CD1a$^+$ DC and RFD9$^+$ macrophages in MSG in which a lymphocytic infiltrate is present suggests the need for further investigation to see if these markers can be helpful in the histopathological diagnosis of Sjögren's syndrome.

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Chapter 4

APOPTOSIS AND APOPTOSIS RELATED MOLECULES IN THE SUBMANDIBULAR GLAND OF THE NOD MOUSE MODEL FOR SJÖGREN'S SYNDROME: LIMITED ROLE FOR APOPTOSIS IN THE DEVELOPMENT OF SIALOADENITIS

submitted

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Abstract

Sjögren's syndrome is an autoimmune disease in which lymphocytic infiltrates develop in the exocrine glands. Pathogenetic aspects of the disease can be studied in the nonobese diabetic (NOD) mouse strain, a spontaneous model for Sjögren's syndrome. Apoptosis may play a role in the initiation phase and in the effector phase of autoimmune diseases. Here, we have examined the role of apoptosis in the development of sialoadenitis in the NOD mouse. Apoptotic cells and the expression of apoptosis related molecules were studied in submandibular glands (SMG) of NOD and NOD-*scid* mice before and following the onset of sialoadenitis. Numbers of apoptotic cells were not increased as compared with control mice, at any age. By immunohistochemistry, we did demonstrate increased expression of Fas, FasL, and bcl-2 on SMG epithelial cells of NOD and NOD-*scid* mice, as early as 3 days of age. By RQ-PCR, also mRNA expression of Fas and FasL was examined in SMG. Low level expression of Fas and FasL mRNA was observed in all mouse strains, from 1 day of age onwards. We conclude that increased protein expression of Fas and FasL on SMG epithelial cells of NOD and NOD-*scid* mice probably indicates a genetically programmed abnormality in these cells that may form a trigger for the development of sialoadenitis in NOD mice. As increased numbers of apoptotic cells were not observed, a role for actual apoptosis in the initiation or effector phase of sialoadenitis in the NOD mouse is unlikely.

Introduction

Sjögren's syndrome is an autoimmune disease with unknown etiology, affecting primarily the salivary and lacrimal glands. In these glands, focal lymphocytic infiltrates develop, which are in part of the patients accompanied by a decreased secretory response (1-3). Spontaneous mouse models for Sjögren's syndrome exist in which different pathogenetic aspects of the disease can be studied. A widely used mouse model for Sjögren's syndrome is the nonobese diabetic (NOD) mouse. In this mouse strain, lymphocytic infiltrates can be detected in the salivary (sialoadenitis) and lacrimal glands (dacryoadenitis) from the age of 10 weeks onwards (4, 5).

The development of sialoadenitis can principally be divided in two phases. An asymptomatic phase in which so far unknown events lead to the activation of autoreactive lymphocytes, followed by a second phase in which lymphocytic infiltrates develop and loss of secretory function can be observed. The cause of the autoimmune reaction may reside in the target organ of the autoimmune response, in the immune system, or in both. Studies in the NOD mouse indicate an important role of the target organ in the initiation of sialoadenitis. First, it has been demonstrated that 18-week-old NOD-*scid* mice exhibit an altered salivary protein composition as compared with control strains (6). Since NOD-*scid* mice lack functional B- and T-lymphocytes (7), the origin of this abnormality most likely resides in the salivary

glands. Second, we have shown that the development of sialoadenitis in the NOD mouse is preceded by the accumulation of dendritic cells into the submandibular glands (SMG) (8). Since dendritic cells are potent antigen presenting cells, involved in the activation of immune responses, their early accumulation into the SMG also points to a local trigger for initiation of sialoadenitis (9-11).

Apoptosis is an important process, involved in maintenance of homeostasis of multicellular organisms (12, 13). It plays a crucial role in morphogenesis and remodeling of tissues during fetal life, in normal tissue turnover and in maintenance of immunological tolerance (13, 14). Although apoptosis has always been considered a way of cell death, not inducing an inflammatory response, it has recently been shown that under certain circumstances apoptotic cells may induce an immune reaction. This may increase susceptibility to, or even result in the development of an autoimmune disease (15-17). For example, accumulation of high numbers of apoptotic cells, due to inefficient clearing or when apoptosis occurs at a high level, may result in the production of autoantibodies to remnants of apoptotic cells, evidence for which has been obtained in mice that are defective in the phagocytosis of apoptotic cells (18, 19). It can also be hypothesized that apoptosis results in the formation of cryptic epitopes of antigens, via cleavage of cellular substrates by enzymes that are activated following induction of apoptosis. These cryptic epitopes may subsequently induce an autoimmune response. Interestingly, induction of apoptosis in a T cell hybridoma results in the formation of 120 kD α-fodrin (20, 21), to which autoantibodies have been detected in serum of patients with Sjögren's syndrome (22, 23). This illustrates that apoptosis may indeed play a role in the pathogenesis of Sjögren's syndrome. Moreover, high numbers of apoptotic cells have been shown to trigger the maturation of dendritic cells *in vitro* (15). This may be mediated by the release of double stranded DNA from dying cells, as this has been shown to induce the maturation of antigen presenting cells (24). So, apoptosis could contribute to the initiation of an (auto)immune reaction via the release of antigens which are normally present in the cell, via the generation of cryptic antigens, or via the maturation of antigen presenting cells which may subsequently activate autoreactive lymphocytes.

In the effector phase of sialoadenitis, apoptosis may contribute to destruction of glandular epithelial cells. In minor salivary glands of patients with Sjögren's syndrome, increased numbers of apoptotic epithelial cells have been detected as compared with controls. In addition, epithelial cells expressed high levels of the apoptosis receptor Fas as well as the death inducing molecule FasL. Infiltrating lymphocytes were shown to express FasL and bcl-2, enabling these cells to induce apoptosis, whilst themselves being protected for the induction of apoptotic cell death (25-28).

The aim of this study is to examine the role of apoptosis in the pathogenesis of sialoadenitis in the NOD mouse. Therefore we studied the presence of apoptotic cells and the expression of apoptosis related molecules (Fas, FasL, and bcl-2) in the SMG by immunohistochemistry. In addition, expression of Fas and FasL was studied by Western blot and by RQ-PCR. To investigate if disturbed apoptosis might contribute to the initiation or to the effector phase of sialoadenitis in the NOD mouse, SMG were studied of pre-autoimmune mice (1 day-

7 weeks) and of mice aged 12-20 weeks, respectively. NOD-*scid* mice were studied in comparison with NOD mice in order to delineate the role of lymphocytic infiltrates.

Materials and methods

Mice and experimental design

Female NOD, NOD-*scid*, and C57BL/10 mice were bred in our own facilities under specific pathogen-free conditions. Specific pathogen-free BALB/c mice were purchased from Harlan (Horst, The Netherlands). Mice were fed standard pellets and water *ad libitum* and were maintained at 22°C +/- 1°C on a 12-hour light/ 12-hour dark cycle. Under these conditions, the incidence of diabetes in NOD mice at 30 weeks of age was 90% in females and 30% in males. SMG of 3-day-old mice were obtained from Hospital Necker, Paris, France, where the mice were housed under the same conditions. Of this age, 10 mice were used of each mouse strain, whereas the other age groups consisted of 5 mice per strain.

Tissue preparation

Mice, aged 3 weeks and older, were killed by asphyxiation with carbon dioxide. Mice, younger than 3 weeks were killed by cervical dislocation. For immunohistochemistry, SMG were removed, embedded in Tissue-tek (Sakura Finetek, Torrance, CA), and snap-frozen in liquid nitrogen. Tissues were stored at -80°C. For RNA analysis, SMG were removed, homogenized in RNAzol^{TM} B, (Campro Scientific, Veenendaal, The Netherlands), and stored at -80°C until further processing. Salivary gland lysates were prepared by homogenization of SMG in ice-cold Hank's buffer (Life Technologies, Paisley, United Kingdom) supplemented with Protease Inhibitor Cocktail (Boehringer Mannheim, Mannheim, Germany), 1 tablet in 10 ml Hank's buffer. The lysates were stored at -80°C.

Immunohistochemistry

Cryostat sections (6 μm) were prepared and mounted on coated microscopic slides. Slides that were used for the immunohistochemical detection of Fas, FasL and bcl-2 were fixed with methanol (-20°C) and aceton (room temperature), and rinsed with phosphate-buffered saline (PBS) (pH 7.8) at room temperature. To block for endogenous biotin-like structures, an avidin/biotin blocking kit was used (Vector Laboratories, Burlingame, CA). Hereafter the slides were incubated with anti-Fas (Ab-1, Calbiochem, Darmstadt, Germany), anti-FasL (C178), or anti-bcl-2 antibody (N19) (both Santa Cruz Biotechnology, Santa Cruz, CA), for one hour at room temperature. As a negative control, the primary antibody was omitted. In addition, the specificity of the antibodies was confirmed by an isotype control (rabbit IgG, Santa Cruz Biotechnology). Subsequently, the slides were incubated for 30 minutes (45 minutes in case of anti-FasL and anti-bcl-2 stainings) with biotinylated goat-anti-rabbit immunoglobulins (Biogenex, San Ramon, CA), diluted 1:50 in PBS/0.1% BSA to which 10% normal mouse serum was added. This was followed by an incubation period of 45 minutes

(30 minutes in case of anti-Fas and anti-bcl-2 stainings) with horseradish peroxidase-conjugated avidin/biotin complex (DAKO, Glostrup, Denmark), diluted 1:100 in PBS after which the peroxidase label was developed by exposure to 0.10% (w/v) diaminobenzidine in acetate buffer (pH 6.0) containing 1% $NiSO_4$ and 0.02% H_2O_2 for three minutes. The slides were counterstained with nuclear fast red [0.1% (w/v) solution in water containing 5% (w/v) $Al_2(SO_4)_3$], dehydrated by an ethanol/xylene series and embedded with Depex mounting medium (BDH, Poole, England). In between all incubations, the slides were rinsed with PBS. The slides were semiquantitatively analyzed by two independent individuals, according to Table 1.

Table 1. Semiquantitative analysis of sections, stained for the presence of Fas, FasL and bcl-2

No positive staining cells	-
Few positive staining cells	-/+
Significant number of positive staining cells	+
High number of positive staining cells	++
Very high number of positive staining cells	+++

Slides that were used for the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL assay) were fixed for 10 minutes in a 4% paraformaldehyde solution (pH 7.4) (Merck, Darmstadt, Germany), at 4°C, and refixated with ethanol/ glacial acetic acid (2:1) at -20°C. Thereafter the sections were presoaked with 50 µl TdT buffer (100 mM sodiumcacodylate, 1 mM cobalt chloride, 0.1 mM dithiothreitol and 0.005% BSA) for 5 minutes at room temperature. Subsequently, 50 µl TdT solution was added containing 20 U TdT (Pharmacia Biotech, Uppsala, Sweden), 20 µM dNTP (Pharmacia Biotech) and 2 µM digoxygenin-labeled dUTP (Boehringer Mannheim, Mannheim, Germany), which was followed by an incubation period of 1 hour at 37°C. As a negative control, TdT was omitted. Following the incubation period, the reaction was stopped by washing for 30 minutes with 2 x concentrated SSC, containing 3 M sodiumchloride and 0.3 M trisodiumcitrate, at 37°C. Thereafter the slides were rinsed with PBS, as in between all incubation steps so far, and subsequently with Tris buffered saline (TBS) (pH 7.6), containing 0.1% (v/v) Triton X-100 and 0.1% BSA. This was followed by an incubation with alkaline phosphatase labeled anti-digoxygenin $F(ab)_2$ fragments (Boehringer Mannheim), 1.5 U/ml in TBS supplemented with 2% fetal calf serum (BioWhittaker, Verviers, Belgium) for 30 minutes at room temperature. Hereafter the slides were placed in a Fast Blue substrate (Sigma, St. Louis, MO) solution for 30 minutes at room temperature, in a dark room. This reaction was stopped by washing for 10 minutes in PBS, after which the sections were counterstained with nuclear fast red (Fluka Chemica, Neu-Ulm, Swiss) and embedded in Kayser's glycerol gelatin (Merck). The sections were evaluated by counting the number of positive staining cells per 7.5 mm^2, by two independent individuals.

Western Blot analysis

Salivary gland lysates were thawed, sonicated twice for 30 seconds and centrifuged at 10000g, 4°C, for 10 minutes, after which the supernatant was carefully removed. Protein

concentration in the supernatant was determined using the Bio-rad protein assay (Bio-rad laboratories GmbH, München, Germany). Per lane, 50 µg of protein was electrophoresed in a 12.5% SDS-polyacrylamide gel. Hereafter, protein was transferred to nitrocellulose (hybond ECL nitrocellulose membrane, Amersham Pharmacia biotech, Little Chalfont, England), blocked for 1 hour in TBS (pH 7.4) supplemented with 0.1% Tween (TBS-T) containing 5% non fat milk and 1% bovine serum albumine. Hereafter, the nitrocellulose membranes were incubated overnight at 4°C with either anti-Fas antibody (0.5 µg/ml, A20, Santa Cruz Biotechnology) or anti-FasL antibody (1 µg/ml, C178, Santa Cruz Biotechnology). The anti-Fas antibody, used in the Western Blot experiments was of a different source than the anti-Fas antibody used for immunohistochemistry, because the latter antibody was not suitable for Western Blot analysis. As a negative control, the primary antibody was substituted by an isotype control (rabbit IgG, Santa Cruz Biotechnology). To control for the specificity of the anti-Fas antibody, the antibody (A20) was neutralized with the corresponding blocking peptide (Santa Cruz Biotechnology) by incubation with a 8-fold excess of blocking peptide for two hours at 4°C, prior to addition to the nitrocellulose filters. The membranes were washed for 1 hour with TBS-T, after which they were incubated with horseradish peroxidase conjugated swine anti-rabbit antibody (2 µg/ml, DAKO) in the presence of 2% normal mouse serum. Blots were developed with chemiluminescence substrate (ECL, Amersham Pharmacia Biotech).

RNA extraction and cDNA synthesis

Total RNA was extracted from SMG tissues that were homogenized in RNAzol^{TM} B, according to the manufacturers protocol. The OD260 and OD280 were measured to determine the yield and purity of the RNA. Target RNA (1 µg) was reverse transcribed using per reaction: 5U AMV-RTase, 2 µl 10 x concentrated AMV RT buffer, 1 µl 20 mM dNTP mix, 2 µl 10 mM spermine/HCl, 1 µl 40 U/µl RNA guard, 0.5 µl 100 OD/ml random hexamers, and 2 µl 100 µg/ml oligo$(dT)_{15}$. This reaction mixture was adjusted with H_2O to a total volume of 20 µl, incubated at 41°C for 1 hour and stored at -80°C. Of each mouse strain, two pools of RNA were used per age group, which consisted of three mice per pool.

Primers and probes

PCR primers and fluorogenic probes for the target genes were designed using the computer program Primer Express, and were purchased from PE Biosystems (Branchburg, NJ). The oligonucleotide sequences of the primers, used for the detection of Fas, FasL, and GAPDH are: Fas 3', ATG CAT CAC TCT TCC CAT GAG A; Fas 5', GGA GGG CAA GAT AGA TGA GAT CA; FasL 3', AAC CCA GTT TCG TTG ATC ACA A; FasL 5', CCA ACC AAA GCC TTA AAG TAT CAT C; GAPDH 3', TTC ACC ACC ATG GAG AAG GC; GAPDH 5', GGC ATG GAC TGT GGT CAT GA. The oligonucleotide sequences of the fluorogenic probes are as follows: Fas, AGT CCA GCT GCT CCT GTG CTG GTA; FasL, CAT TTA ACA GGG AAC CCC CAC TCA AGG T; GAPDH, TGC ATC CTG CAC CAC CAA CTG CTT AG. The fluorogenic probes contained a reporter dye (FAM) covalently attached

to the 5' end and a quencher dye (TAMRA) covalently attached to the 3' end. Extension from the 3' end was blocked by attachment of a 3' phosphate group.

PCR amplification

PCR reactions were performed in the ABI-prism 7700 sequence detector, which contains a Gene-Amp PCR system 9600 (Perkin Elmer/ Applied Biosystems, Foster City, CA). Reaction conditions were programmed on a Power Macintosh 7200. PCR amplifications were performed in a total volume of 50 µl, containing 2 µl cDNA sample, 25 µl 2 x concentrated Taqman® Universal PCR Master Mix (PE Biosystems, Branchburg, NJ), 900 nM of each primer (for Fas and GAPDH detection). Each reaction also contained 200 nM of the corresponding detection probe (for Fas and GAPDH detection). For the detection of FasL expression, 1200 nM of each primer and 250 nM of the detection probe were used. PCR amplification reactions were performed in duplicate wells, using the following conditions: 2 min at 50°C and 10 min at 95°C, followed by a total of 50 two-temperature cycles (15 s at 95°C and 1 min at 60°C).

Results

Presence of apoptotic cells in submandibular glands of different mouse strains

Apoptotic cells, detected by the TUNEL assay were counted per 7.5 mm^2 glandular tissue. In all mouse strains, at all ages, apoptosis was mainly confined to acinar epithelial cells, hardly any apoptotic cells were present in ductuli (Fig. 1A). At the age of 3 days, increased numbers of apoptotic cells were detected in SMG of NOD, NOD-*scid* and C57BL/10 mice, as compared with 5 and 20 weeks of age (Fig. 2). In NOD-*scid* mice, this difference was less pronounced as compared with NOD and C57BL/10 mice. At three days of age, no significant difference was observed between numbers of apoptotic acinar epithe-

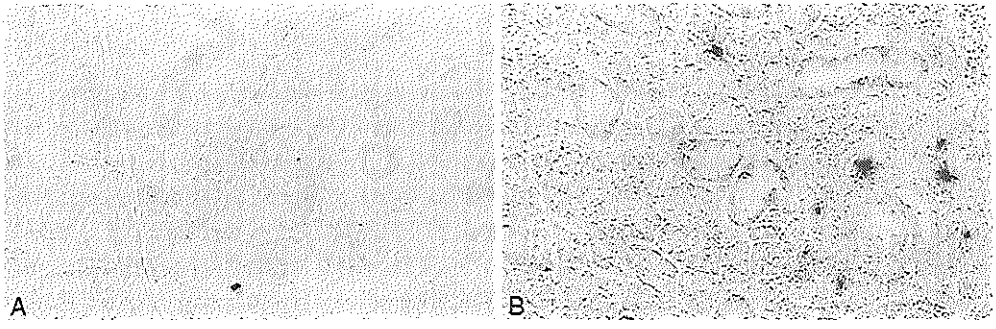

Figure 1
Apoptotic cells, present in the SMG of the NOD mouse, as detected by the TUNEL assay. A: 5-week-old mouse B: lymphocytic infiltrate, in SMG of 20-week-old mouse (*250).

lial cells in the SMG of NOD and control mice. Also in glands of 5- and 20-week-old mice, numbers of apoptotic acinar epithelial cells were comparable among the different mouse strains. Within the lymphocytic infiltrates in SMG of 20-week-old NOD mice, apoptotic cells could also be observed (on average 5 apoptotic cells per infiltrate) (Fig. 1B).

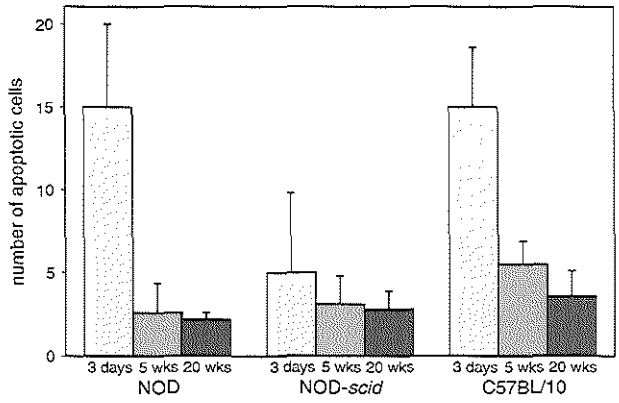

Figure 2
Numbers of apoptotic cells, present in SMG of different mouse strains (3 days till 20 weeks of age) as determined by the TUNEL assay. Numbers were counted per 7.5 mm^2 glandular tissue and are expressed +/- standard deviation.

Immunohistochemical detection of Fas, FasL and bcl-2

SMG of mice, aged 3 days, 5, and 20 weeks were studied for the expression of Fas, FasL and bcl-2 by immunohistochemistry. In glands of 3-day-old mice, it was difficult to discriminate between positive staining, localized on acinar or ductal epithelial cells. For this reason the results are described as positive staining in the parenchyma of the gland. At the age of 5 and 20 weeks, a distinction between staining on acinar and ductal epithelial cells was made. At 3 days of age, increased expression of Fas was observed in SMG of NOD and NOD-*scid* mice as compared with control mice (C57BL/10) (Table 2). This difference was more pronounced at 5 and 20 weeks of age, when Fas expression was not detected in the SMG of the C57BL/10 mouse, whereas in the NOD mouse, clear positive staining was observed on acinar and ductal epithelial cells (Table 2, Figs. 3, A and B). On acinar cells of NOD-*scid* mice, intermediate level of Fas expression was detected at the age of 5 and 20 weeks, whereas on ductal cells, Fas expression was only observed at 20 weeks of age.

Expression of FasL was not detected in the SMG of the C57BL/10 mouse, at any age. In the SMG of NOD and NOD-*scid* mice on the other hand, FasL expression was observed at all ages (Table 3, Figs. 3, C and D). At 5 and 20 weeks of age, FasL was observed on acinar and ductal epithelial cells, this expression was higher in NOD as compared with NOD-*scid* mice. Bcl-2 expression was detected in SMG of all mouse strains, from the age of 3 days

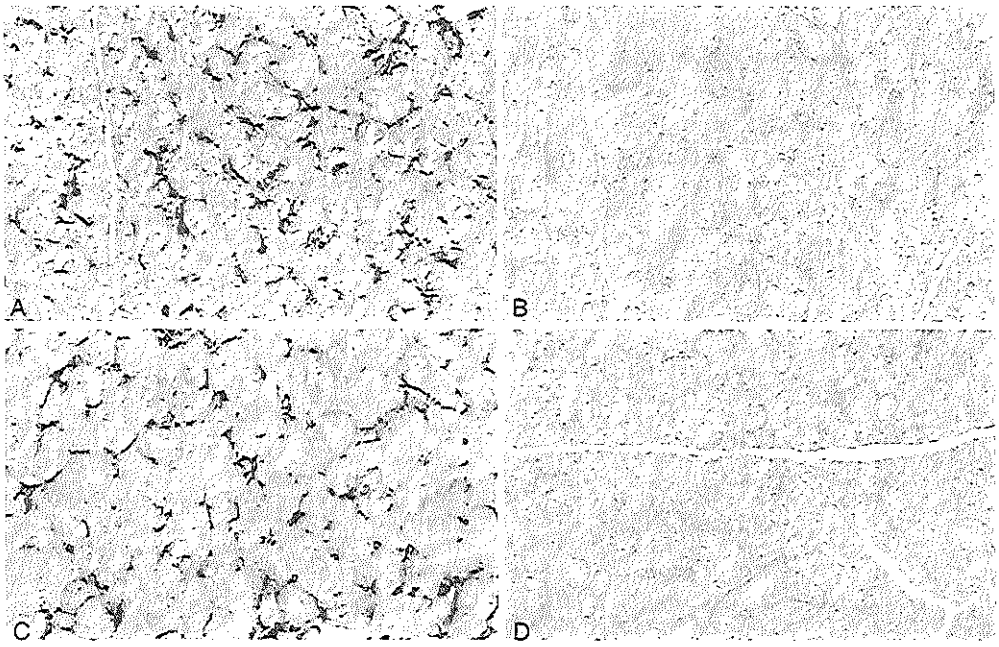

Figure 3
Immunohistochemical detection of Fas and FasL expression in SMG of different mouse strains, at 5 weeks of age. A,B: Expression of Fas in the SMG of the NOD (A) and C57BL/10 (B) mouse strains. C,D: Expression of FasL in the SMG of the NOD (C) and C57BL/10 (D) mouse strains (*250).

till 20 weeks on, but the expression level varied among the different mouse strains (Table 4). Highest expression was found in the SMG of the NOD mouse, at all ages. Expression of Fas, FasL and bcl-2 was also examined in the SMG of the BALB/c mouse. Results obtained in this mouse strain were similar to results we found in C57BL/10 mice (results not shown). In SMG of NOD mice, lymphocytic infiltrates were detected at the age of 20 weeks. Infiltrating cells present within these infiltrates expressed high levels of Fas, FasL, and bcl-2 (results not shown). So, increased expression of Fas, FasL and bcl-2 was observed on SMG epithelial cells of 3-day through 20-week-old NOD and NOD-*scid* mice as compared with age matched control mice.

Fas and FasL protein expression in submandibular gland lysates

Lysates from SMG of 3 and 18-week-old NOD, NOD-*scid* and C57BL/10 mice were subjected to Western Blot analysis to study expression of Fas and FasL expression. At three weeks of age, Fas expression was observed in SMG of all mouse strains (Fig. 4A). No major difference was observed between the different mouse strains. Slight variations observed among the different mouse strains are probably due to variation in the amount of protein, loaded on the gel. A similar picture was observed at 7 weeks (results not shown) and 18 weeks of age, at which comparable levels of Fas expression were observed in SMG of all

Table 2. Fas protein expression in submandibular glands of different mouse strains

	Parenchyma	Acinar epithelial cells		Ductal epithelial cells	
	3 days	5 weeks	20 weeks	5 weeks	20 weeks
NOD	+/++	++	+++	+	++
NOD-*scid*	+/++	-/+	+	-	+
C57BL/10	+	-	-	-	-

Table 3. FasL protein expression in submandibular glands of different mouse strains

	Parenchyma	Acinar epithelial cells		Ductal epithelial cells	
	3 days	5 weeks	20 weeks	5 weeks	20 weeks
NOD	+	++	++	+	++
NOD-*scid*	+	+/-	+	-/+	+
C57BL/10	-	-	-	-	-

Table 4. Bcl-2 protein expression in submandibular glands of different mouse strains

	Parenchyma	Acinar epithelial cells		Ductal epithelial cells	
	3 days	5 weeks	20 weeks	5 weeks	20 weeks
NOD	+/++	++	+++	+	++
NOD-*scid*	+/++	+	+	+/-	+/-
C57BL/10	+/-	+/-	+	-/+	+/-

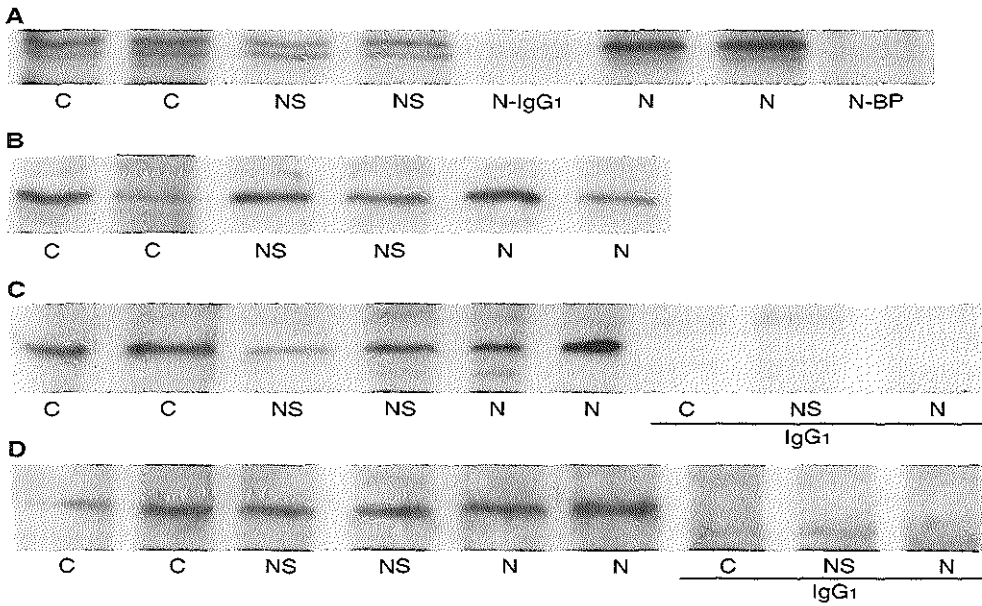

Figure 4
Western blot analysis of Fas (A,B) and FasL (C,D) expression in SMG lysates of NOD, NOD-scid and C57BL/10 mice aged 3 weeks (A,C) and 18 weeks (B,D). C: C57BL/10, NS: NOD-*scid*, N: NOD. IgG1: IgG1 isotype control as primary antibody, BP: Preincubation of primary antibody with blocking peptide.

mouse strains (Fig. 4B). No signal was observed when the anti-Fas antibody was substituted by an isotype control antibody. In addition, preincubation of the primary antibody with a Fas blocking peptide inhibited the positive signal, observed in the samples (Fig. 4A). Expression of FasL was found in SMG lysates of 3 and 18-week-old NOD, NOD-*scid* and C57BL/10 mice (Fig. 4, C and D). Again, no difference was observed between the mouse strains or between the two age groups. Substitution of the primary antibody with an isotype control resulted in absence of a positive signal (Fig. 4, C and D).

Quantification of Fas and FasL mRNA by RQ-PCR

RQ-PCR was performed to examine and quantitate Fas and FasL mRNA expression in SMG of the different mouse strains. A standard curve was generated in each experiment in which the threshold cycle was plotted against the starting quantity of input cDNA. This curve was used to deduce the starting quantity of the individual samples, in arbitrary units. To standardize for the amount of RNA which was used in the reverse transcriptase reaction, values obtained in experiments in which Fas or FasL expression was examined were divided by the corresponding values for GAPDH expression. In Tables 5 and 6, the expression levels of Fas and FasL (average of the two pools) in the different mouse strains, at different ages are mentioned. These levels are corrected for the amount of input RNA used to generate cDNA. The C_t value (threshold cycle) is a measure for the amount of template in the sample. C_t values for Fas and FasL were high as compared to C_t values for GAPDH (5-7 C_t higher in case of

Table 5. Expression of Fas mRNA in submandibular glands of different mouse strains*

	1 day	3 weeks	7 weeks	12 weeks	18 weeks
NOD	1.5	1.0	0.5	1.0	0.8
NOD-*scid*	1.2	1.1	0.9	0.7	0.9
C57BL/10	0.9	1.8	0.7	0.3	0.5
BALB/c	0.9	0.3	0.6	0.7	0.8

*: Expression levels are corrected for GAPDH mRNA expression

Table 6. Expression of FasL mRNA in submandibular glands of different mouse strains *

	1 day	3 weeks	7 weeks	12 weeks	18 weeks
NOD	0	0.3	0.1	0.5	0.5
NOD-*scid*	0	0.3	0.1	0	0.2
C57BL/10	0	0.4	0.3	0	0.1
BALB/c	0	0	0	0.1	0.5

*: Expression levels are corrected for GAPDH mRNA expression

Fas, 7-10 C_t higher in case of FasL, with similar amounts of input cDNA in the RQ-PCR reaction), which means that 5 to 10 extra amplification cycli are needed in order to detect a signal for Fas or FasL than the number of cycli needed to detect GAPDH.

It can be concluded that Fas mRNA expression occurs in all mouse strains, from 1 day through 18 weeks of age, albeit at low levels. Taken into consideration the variation among the different age groups, and among the two control strains, no major differences are detected between the NOD and NOD-*scid* on the one hand, and the control strains on the other

hand, at any age. Furthermore, between the individual mouse strains, no significant difference in Fas mRNA expression is observed in time. FasL mRNA expression is absent in all mouse strains at 1 day of age. At 3 and 7 weeks of age, expression is low in NOD, NOD-*scid* and C57BL/10 mice, and still absent in BALB/c mice. Whereas in the NOD-*scid* and C57BL/10 mice, FasL mRNA expression remains low at 12 and 18 weeks of age, in the NOD mouse it is increased.

Discussion

In this study we demonstrated by immunohistochemistry increased expression of the pro-apoptotic molecules Fas and FasL in SMG epithelial cells of mice of the NOD strain as compared with control mice. This increased expression was already observed as early as three days of age, and was not accompanied by increased numbers of apoptotic epithelial cells. This may be due to the concurrent increased expression of the anti-apoptotic bcl-2 on the salivary gland epithelial cells, which was most obvious in the NOD mouse. Furthermore, the final outcome of whether a cell undergoes apoptosis or not can be influenced by a list of factors that is ever increasing, of which we studied only three factors (29, 30).

A major concern of this study is that increased expression of Fas and FasL was only identified by immunohistochemistry. Western Blot analysis of SMG lysates revealed Fas and FasL expression in 3 to 18-week-old NOD, NOD-*scid* mice, and C57BL/10 mice, without quantitative differences among the mouse strains. The discrepancy might be due to the presence of Fas and FasL protein in intracellular compartments, not bound to membrane structures, whereas in NOD and NOD-*scid* mice, membrane expression does occur. In cytotoxic T lymphocytes, it has been demonstrated that FasL mediated cytotoxicity, induced upon T cell receptor mediated activation, could be blocked by an inhibitor of intracellular transport, but not by inhibition of protein synthesis or DNA transcription (31). Increased expression of Fas has been observed on UV-B irradiated human peripheral blood lymphocytes, which was not dependent on protein synthesis (32). These results imply the existence of preformed FasL and Fas protein in the cytoplasm of cytotoxic T lymphocytes and peripheral blood lymphocytes, respectively, which, upon activation by T cell receptor triggering or exposure to UV-B, would be translocated to the cell membrane. This mechanism had already been proposed for cytotoxic T lymphocytes before (33). Our results imply that it is particular on the level of membrane expression of Fas and FasL that NOD and NOD-*scid* mice differ from C57BL/10 mice. In contrast, the production of Fas and FasL between the mouse strains would not be different, as indicated by equal mRNA expression and similar protein levels, detected by Western Blot analysis. Membrane expression of Fas and FasL would not necessarily serve the physiological function as to result in the induction of apoptosis, but may be the outcome of dysregulated gene expression, resulting in activation of the cells. The nature of the signals for such expression remains highly speculative, but could include dysregulated expression of extracellular matrix components and/or of enzymes capable of degrading these components.

Loss of attachment to the extracellular matrix can result in apoptosis in many cell types, a process named anoikis (34). In a human umbilical vein endothelial cell line (HUVEC), this process has recently been demonstrated to depend on Fas ligation. Detachment of HUVEC resulted in a three-fold increase in the cell surface levels of Fas (35). Although increased mRNA expression was also observed, other mechanisms were not studied, thus not excluding that Fas protein, present in intracellular vesicles may be translocated to the cell membrane upon activation. Interestingly, reduced expression of the matrix metalloproteinases MMP-2 and MMP-9 has been described in the SMG of neonatal NOD mice, an age at which membrane expression of Fas and FasL was already observed, whereas expression of both enzymes was increased at 3 weeks of age (36).

Kong *et al* described increased numbers of apoptotic epithelial cells in SMG of 18-week-old NOD and NOD-*scid* mice as compared with control mice and suggested an important contribution of apoptosis to the development of sialoadenitis (37). In glands of 8-week-old mice, similar numbers of apoptotic cells were detected. By immunohistochemistry and on mRNA level, constitutive expression of FasL in both NOD, NOD-*scid* as well as in BALB/c mice, aged 8 and 18 weeks was described. Fas protein expression on the other hand was restricted to SMG of 18-week-old NOD and NOD-*scid* mice, but Fas mRNA expression was low. Our study does not support their findings. The reason for the discrepancy between their and our results may reside in a different source of the antibodies, used for immunohistochemistry. Furthermore, differences in housing conditions of NOD mice may influence the outcome of experiments in different laboratories, although this is unlikely to result in major discrepancies other than kinetics of the disease.

In conclusion, our results have important implications with regard to the potential role of apoptosis in the development of sialoadenitis. Whereas a role of apoptosis in the effector phase of sialoadenitis in the NOD mouse was suggested following the observations of Kong et al (37-39), our results do not support this. In addition, in our experiments we did not find evidence for a role of apoptosis in the initiation phase of sialoadenitis. We prefer the idea that disturbed membrane expression of Fas, FasL and bcl-2, already apparent as early as 3 days of age and continuing till 20 weeks of age, may reflect a genetically programmed abnormality intrinsic to the SMG, as has been suggested for other abnormalities that were described in the SMG of the NOD mouse (6). The abnormalities may be hallmarks of disturbed homeostasis of the NOD SMG, which may increase susceptibility to the development of sialoadenitis.

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Chapter 5

ABNORMAL ORGANOGENESIS IN SALIVARY GLAND DEVELOPMENT MAY INITIATE ADULT ONSET OF AUTOIMMUNE EXOCRINOPATHY

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Abstract

Objectives. Salivary gland organogenesis was evaluated in NOD mice, an animal model for autoimmune exocrinopathy, to determine when disease onset is first present in the target tissues.

Methods. Submandibular glands were removed for histological, immunohistochemical, and biochemical evaluation from neonatal NOD and congenic strains as well as healthy control C57BL/6 mice.

Results. Histomorphological analyses of neonatal submandibular glands, the primary target for autoimmune exocrinopathy at 1 day *postpartum*, revealed delayed morphologic differentiation during organogenesis in autoimmune-susceptible NOD mice when compared to non-susceptible C57BL/6 mice. Acinar cell proliferation was reduced, while expression of Fas, FasL, and bcl-2 were increased. Throughout the pre-weaning period (21 days) submandibular glands from NOD and NOD congenic strains demonstrated increased matrix metalloproteinase (MMP)-2 and MMP-9 activity. Substitution of two susceptibility alleles (*Idd3* and *Idd5*) in NOD mice resulted in an hierarchical and additive reversal of delayed organogenesis, elevated MMP-9 activity, and aberrant expression of parotid secretory protein (PSP).

Discussion. NOD-derived mice whose submandibular glands showed normal organogenesis did not progress to develop autoimmune exocrinopathy. Altered organogenesis of target tissue may therefore provide a cellular microenvironment capable of activating autoimmunity.

Introduction

The development of the submandibular glands involves the orchestrated expression of extracellular matrix (ECM) molecules that direct the morphogenesis and cytodifferentiation of the epithelium through the regulation of both proliferation and apoptosis. These events are highly regulated and coordinated, both temporally and spatially (1-3). Full expression of proper morphogenesis and cytodifferentiation is determined by the action of the matrix degrading enzymes, the matrix metalloproteinases (MMPs), that remodel components of the ECM (4-6). The various roles that collagens and specific MMPs (6, 7) play in the morphogenesis of the exocrine tissues are highlighted by the synthesis and deposition of type I and type III collagens which are required for branching morphogenesis in the salivary glands (8-10). In contrast, type IV collagen appears to play a role in the regulation of salivary gland secretory cell differentiation (11).

Sjögren's syndrome is a human autoimmune disease characterized by the loss of exocrine function presumed to result from active lymphocytic destruction of salivary and lacrimal glands (12, 13). In addition to the primary site of Sjögren's syndrome involving the salivary and lacrimal glands, additional exocrine tissues may become involved including skin, lungs, gastrointestinal tract, and vaginal tissues (12). Diagnosis of the disease often

includes the detection of peri-ductal foci of infiltrating lymphocytic populations in the minor salivary glands determined through the histopathological analysis of a labial gland lip biopsy. Serological evaluations are used to identify the presence of rheumatoid factor, elevated immunoglobulin levels and specific anti-nuclear antibodies to SS-A/Ro and SS-B/La (13). Xerostomia (dry mouth) and xerophthalmia (dry eye) are assessed by specific tests for changes in exocrine gland flow rates and biochemical changes in protein composition.

The etiology of autoimmune diseases, in general, has remained elusive despite significant effort to identify genetic, viral, and hormonal mechanisms of initiation and this remains true for Sjögren's syndrome (12, 13). The availability of an autoimmune murine model, the NOD mouse and its congenic partner strain NOD.B10.$H2^b$, two strains exhibiting a temporal lymphocytic infiltration of the exocrine tissues that correlates with loss of secretory function, has permitted detailed studies into the pathogenesis underlying Sjögren's syndrome (14-17). In addition, previous studies (18-20) in the congenic immunodeficient NOD-*scid* mouse have provided evidence for the involvement of genetically programmed non-immune factors contributing to the loss of differentiated function or tissue homeostasis prior to onset of detectable autoimmunity.

Although the *scid* mutation prevents the spontaneous development of both sialoadenitis and dacryoadenitis in these mice, a number of biochemical markers of differentiated function are still diminished or aberrantly processed in exocrine glands in the absence of detectable lymphocytic infiltration or loss of secretory function (18-20). Elevated levels of caspase and MMP activity accompany morphological loss of submandibular gland acinar cell structures (19, 21). Aberrant proteolytic processing may generate cryptic antigens, priming the immune system for an autoimmune response (22, 23). These studies, together with results of studies involving other strains, e.g., NOD.Igμ^{null}, have led to the concept that autoimmune exocrinopathy in NOD mice progresses in two phases; an asymptomatic phase, in which epithelial cells of exocrine tissues undergo dedifferentiation accompanied by elevated activation of biomarkers for apoptosis, occurs between 8-12 weeks of age, and a second phase, in which autoaggression is mounted against target organ autoantigens resulting in the clinical presentation of loss of secretory function, taking place at 14-16 weeks of age (16, 18, 19, 24).

The salivary glands of mice are functionally immature at birth. During the first three weeks of age, the salivary glands undergo further morphodifferentiation, acquiring the capacity to express and synthesize salivary-specific gene products. Finally, there is a functional coupling of the autonomic nervous system prior to weaning (25, 26). Since the physiological and biochemical alterations in non-immune factors observed prior to onset of autoimmune exocrinopathy resemble the functional status of pre-weaning organogenesis, we have evaluated submandibular gland development in neonatal mice *postpartum*.

Materials and methods

Animals

Neonatal mice at 24 hrs of age and subsequent times of 3, 8, 14, and 21 days were obtained from the breeding of CD1, C57BL/6, BALB/c, NOD/Lt, NOD.B10.$H2^b$, and NOD.B10.$H2^b$-*scid*. NOD.B6*Idd3*, NOD.B10*Idd5* and NOD.B6*Idd3*.B10*Idd5* mice were used at 24 hrs, 3 weeks and 20 weeks of age. The NOD.B10.$H2^b$-*scid* mouse was generated by crossing the NOD-*scid* with a NOD.B10.$H2^b$ strain. The F1 heterozygous MHC and *scid* loci were backcrossed with the F2, screened by FACS cell sorting for T- and B-lymphocytes isolated by a tail bleed and microsatellite typing of the homozygous $H2^b$ on the NOD background covering the D17Mit68 and D17Mit34 regions (27). All animal procedures were carried out with the approval of the University of Florida Animal Welfare Committee.

Preparation of tissue for histological and immunohistochemical evaluation

Freshly excised neonatal submandibular glands were fixed in 10% phosphate-buffered formalin. Additional tissue samples were taken for kidney, liver, pancreas, lung and heart. Each tissue was embedded in paraffin, sectioned in 5 μm thick sections, and stained with hematoxylin/eosin (14). The stained sections were viewed by light microscopy at 200X magnification.

For immunohistochemical detection of Fas, FasL and bcl-2, frozen sections were placed on slides treated with 0.01% poly-L-lysine followed by fixation in methanol/acetone at -20°C and acetone at room temperature (3 times for 5 sec for each wash solution). Slides were incubated for 1 hr with primary antibody to Fas (Ab-1, Calbiochem, Darmstadt, Germany), FasL (1:500, Santa Cruz, CA), and bcl-2 (1:800, Santa Cruz, CA) followed by a biotin-labeled goat anti-rabbit second antibody conjugate (Biogenex, San Ramon, CA). The slides were incubated with a peroxidase conjugated avidin/biotin complex (1:100, Strept ABComplex, DAKO, Glostrop, Denmark). Development was performed by exposure to 0.01% di-amino-benzidine and counter stained with nuclear fast red. Sections were viewed by light microscopy at 200X magnification.

Labeling indices (LI) for proliferation of epithelial cells was determined by the intraperitoneal injection of bromodeoxyuridine (BrdU) (50 mg/kg) two times at one hr intervals prior to killing Submandibular glands were isolated, cleaned of connective tissue and lymph nodes, embedded, and sectioned as above. BrdU incorporation was assessed by detection of an avidin-biotin complex of anti-BrdU antibody (1:200) coupled with a goat anti-mouse secondary antibody (1:100 dilution). The slides were counterstained with hematoxylin for tissue contrast. The LI represents stained positive cells (acini and duct cells) relative to the total number of cells under view and are expressed as mean ± standard error for 3 separate litters for each group. Statistical significance was determined by use of computer software equipped with Student *t* test analysis.

RNA isolation and RT-PCR detection of ECM, MMP and salivary specific mRNA products

mRNA from a litter of pups was isolated using the Micro-FastTrack kit (Invitrogen, San Diego CA). Copy DNA was synthesized from 1 μg of RNA in a standard 20 μl reverse transcriptase reaction, and the PCR reaction was subsequently performed using the RT-PCR kit (Perkin Elmer, San Francisco, USA). The amplification conditions were 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min in a Biometra thermocycler for 25 cycles. The primer sets used were as follows: β-actin, forward TGA AGG TCG GTG TGA AAA CGG ATT TGG C, reverse CAT GTA GGC CATG AGG TCC ACC AC; EGF, forward TAA GCC GAG ACC GGA AGT ACT, reverse AGT CTG TTC CAT CAA ATG CA; PSP forward ATG TTC CAA CTT GGA AGC C, reverse GAG GGC AAG TTG TAC CTG; fibronectin, forward CCG GGT TCT GAG TAC ACA GTC, reverse GGA GGG TCT CTT CAC CAG GGA; αV integrin, forward CGC CAA GTT GCT TGC AGA TCA C, reverse ATC ACC AGC ACG GTG GTG AAC; Collagen IV, forward GGA CAA GCA GGC TTT CCT GGA, reverse GGG ACC GGA AGG ACC TGT CGT; TIMP-2, forward GAG CCA AAG CAG TGA GCG AG, reverse GGT ACC ACG GCG AAG AAC CAT. Primer sequences were derived from GenBank and analyzed using the primer generation program from MacVector (Kodak, Rochester, NY). Primer pairs were synthesized by the University of Florida Oligonucleotide Sequencing Core.

Densitometric analyses of band intensities was performed using an Hewlett-Packard HP IIcx flatbed scanner, coupled to a computer equipped with NIH image and Adobe analyses software. The log ratio of band intensities within each lane was measured relative to the intensities of β-actin controls. All values are expressed as the mean ± standard error.

Measurement of MMP-9 activity in salivary gland lysates

Submandibular gland lysates were prepared from the pool of glands isolated from a liter of pups. The MMP-9 collagenase activity in neonatal submandibular glands was measured using a Chemicon (Temecula, CA) assay kit. Fluorescent activity was measured using a Perkin Elmer LS-3B Fluorescence Spectrometer. A unit of activity is defined as mg of tissue lysate required to cleave 1.0 μg FITC-labeled type IV collagen substrate/min, based on the fluorescence intensity using 520 nm (Em) and 495 nm (Ex). Statistically significant differences, relative to the MMP-9 activity of CD1 control mice are reported as $p < 0.05$.

Results

Histological analysis of salivary gland morphogenesis

To gain a better understanding of the genetically programmed factors regulating neonatal salivary gland development, we evaluated glandular morphogenesis and cytodifferentiation, *postpartum*, in several strains of mice. These included BALB/c, CD1, C57BL/6, NOD.B10.$H2^b$ and several NOD-derived strains exhibiting characteristics of primary and

secondary autoimmune exocrinopathy (Sjögren's syndrome) (28). Neonatal submandibular gland morphology at day 1 *postpartum* revealed that NOD strains show more distinct septation of lobular structure than control strains of mice (Fig. 1A). The interlobular gaps of CD-1 were not as prominent as in NOD mice. BALB/c and C57BL/6 mice had similar glandular morphology to control CD1 (data not shown). H&E stained neonatal gland tissues (Fig. 1B) showed reduced acinar cell populations, wide connective septa, and less organized lobules in NOD mice as compared to CD1 controls. Computer-assisted morphometric analyses indicated a ratio of 9:1 acinar to ductal cells for control and NOD mice. However, the acinar cell volumes were 17% to 28% greater in NOD.B10.$H2^b$ and NOD/Lt, respectively, when

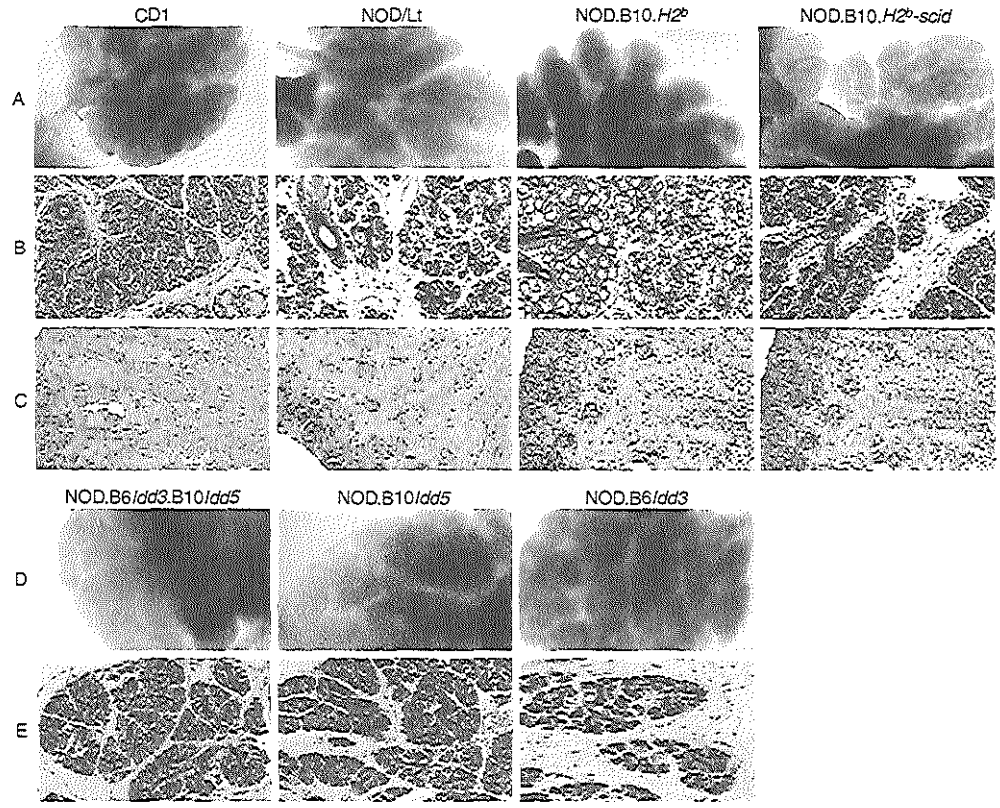

Figure 1
Morphological differences in the neonatal submandibular glands of NOD mice. The submandibular gland was identified by gross morphology in CD1 and NOD congenic strains in mouse litters killed 24 hr after birth, using a Zeiss dissecting microscope. BALB/c and C57BL/6 mice were evaluated with morphology similar to that observed in the CD1 mice. A, gross morphology of submandibular glands. Magnification, 100X. B, detection of histological differences between NOD congenic strains and control mice. C, H & E stained histology of the kidney, a tissue free of autoimmune targeting in Sjögren's syndrome-like pathophysiology in NOD mice. D and E, submandibular gland morphology of NOD congenic mice carrying the autoimmunity resistance alleles of *Idd3*, *Idd5* or the combination of *Idd3* and *Idd5* of C57BL mice (28).

compared to CD1 mice ($p<0.05$). Ductal cell volumes were similar between the three strains.

To examine whether these differences are limited only to the submandibular glands, other organs, such as kidney, heart, liver, spleen, pancreas, and thymus, from the same groups were harvested and compared histologically at 1, 4, 8, 16, and 20 days of age. There were no detectable strain specific abnormalities in any of these organs, as represented by the kidney (Fig. 1C). However, similar ongoing studies in the pancreas of NOD mice, which also serves as a model for type 1 insulin-dependent diabetes, has detailed differences in the islet cell development during the neonatal period when compared to control mice (Pelegri et al., unpublished observations). Interestingly, the submandibular glands of NOD, NOD.B10.$H2^b$ and NOD.B10.$H2^b$-*scid* mice at an early age contained an unidentified mononuclear cell infiltrate (CD11c and F4/80 negative). A similar population has been identified in fetal and neonatal pancreas from humans and mice (29-32). Despite these early deficiencies, by eight days after birth, the morphology of the submandibular gland was similar to the control strains examined, and the infiltrates had disappeared.

Epithelial cell proliferation rates

To evaluate the possible differences in cell proliferation rates in submandibular gland development of NOD and control mice, we investigated the incorporation of BrdU, a thymidine analogue, into DNA of 1-day-old mice and examined glandular epithelial cells subsequently with immunohistochemistry. NOD/Lt and NOD.B10.$H2^b$-*scid* had substantially lower BrdU labeling indices than controls (0.077 ± 0.029 and 0.026 ± 0.008, vs. 0.180 ± 0.014; Table 1) suggesting a reduction in total cell proliferation rates in NOD strains (Fig. 2). The *scid* mutation of NOD mice disrupts a DNA repair enzyme which may in part account for the low rate of proliferation observed in the NOD.B10.$H2^b$-*scid* mice. However, by one week after birth, the rates of acinar cell proliferation were similar to control CD-1 and C57BL/6 mice (Table 1).

Evaluation of factors involved in programmed cell death

Table 1. Cell proliferation rates determined by BrdU labeling indicies*

	CD1	NOD/Lt	NOD.B10.$H2^b$-*scid*
1 day	0.180 ± 0.014	0.077 ± 0.029^a	0.026 ± 0.008^b
7 day	0.367 ± 0.038	0.391 ± 0.49	0.307 ± 0.40

*The tissue area was divided into 4 quadrants for counting the number of labeled nuclei. All values represent the mean ± standard error for 3 glands. $^a p<0.001$ and $^b p<0.0001$.

Since programmed cell death (PCD) is an important mechanism in organogenesis (in addition to proliferation), changes in this process may contribute to the development of autoimmunity. To investigate the role of PCD during development of the submandibular gland *postpartum*, the expression of Fas, FasL, bcl-2, and the presence of TUNEL$^+$ glandular epithelial cells were analyzed in 3-day-old mice. The number of TUNEL$^+$ cells was similar in NOD/Lt and control mice, indicating that there is no major difference in the number of apoptotic cells between these strains of mice. Increased distinct staining patterns, detected

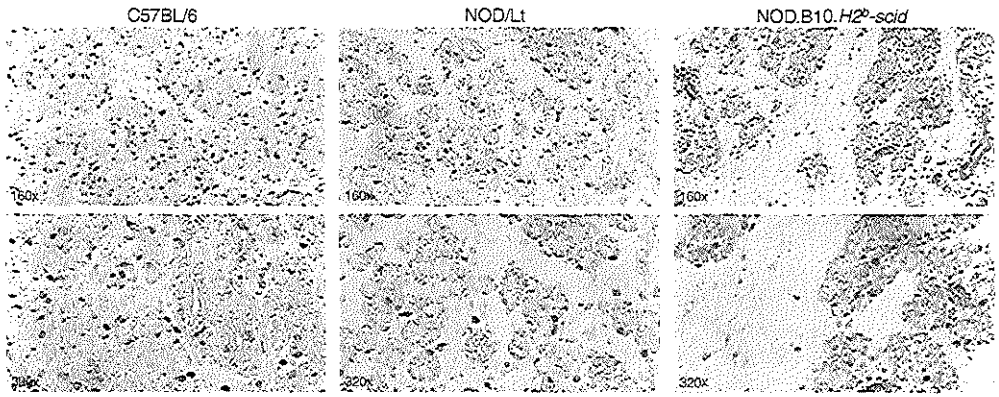

Figure 2
Analysis of cellular proliferation rates of 1-day-old neonatal submandibular glands by BrdU staining. The increased septation described for the NOD strains is again clearly visible at the two magnifications depicted (160X and 320X). Replicating cells were stained using an alkaline phosphatase substrate followed by hematoxylin counterstain for tissue contrast. Note the structural differences evident in NOD strains with the presence of increased septation of the lobules and disorganized acinar components of the lobular structures.

through immunohistochemical analyses of Fas, FasL, and bcl-2, were evident in 3-day old NOD/Lt mice compared to control C57BL/6 mice (Fig. 3). Bcl-2 was detected in most of the cells of the neonatal tissue for both strains. The staining pattern for FasL and Fas was confined to basal cells of the ductal structures and other epithelial cells in NOD strains, while very little staining was observed in C57BL/6 mice (Fig. 3).

The elevated levels of expression of molecules regulating apoptosis in NOD salivary glands may represent an alteration in normal growth and differentiation. The absence of increased PCD, despite the expression of Fas and FasL, could be attributed to the elevated expression of the survival factor bcl-2. In an analogous situation, members of the bcl-2 family play an important role in overriding PCD in the developing mammary gland, suggesting that these proteins are important in the development of exocrine tissues (33).

Expresion of developmental salivary-specific proteins

An assessment of epithelial cell cytological differentiation was performed using markers of salivary gland development. Epidermal growth factor (EGF), a product of the ductal cells of the submandibular gland (14), as well as parotid secretory protein (PSP) (19, 20), a product of the acinar cells of neonatal submandibular glands but whose expression is terminated in rodents by 5 days *postpartum*, were evaluated. Semi-quantitative RT-PCR for EGF and PSP showed no significant difference in the steady state expression levels of mRNA transcripts at the time of birth (Fig. 4 and results not shown). The steady state mRNA concentration for PSP in C57BL/6 and CD1 mice (data not shown for this strain), as well as in NOD/Lt mice, was reduced significantly in submandibular glands prepared from 3-week-old animals relative to that detected in the neonates (Fig. 4). Normal expression of growth factors, such

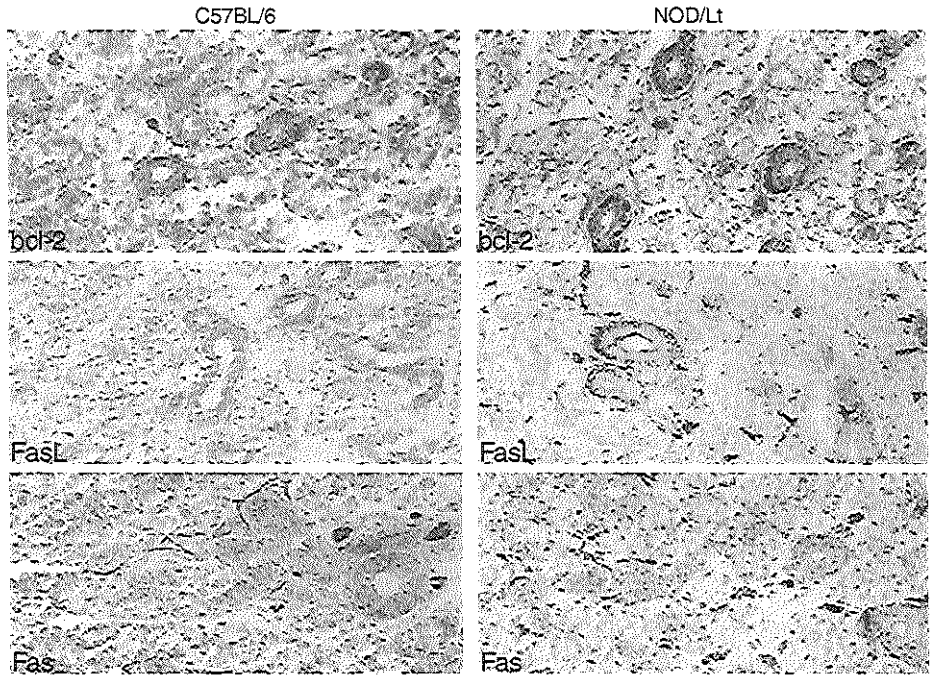

Figure 3
Immunohistochemical staining for Fas, FasL and bcl-2 in 3-day-old C57BL/6 and NOD/Lt mice (n = 10). Similarly distributed increased distinct staining patterns are evident around the ductal epithelium of NOD mice stained with Fas and FasL. Bcl-2 appears to have increased staining over both the ductal and epithelial cell structures. Arrows indicate staining around ductal structures in the tissue sections.

as EGF, may explain why normal glandular structure is achieved by the end of 8 days of age despite the retarded early growth pattern. The steady state mRNA expression levels of integrins, the extracellular matrix molecules (fibronectin and collagen IV), and tissue inhibitors of metalloproteinase (e.g., TIMP-2) were similar in NOD/Lt and C57BL/6 controls at 1 day *postpartum* (results not shown). Similar steady state levels of PSP mRNA were detected in CD1, C57BL/6 and NOD/Lt mice. Using Western blot detection of protein expression, both C57BL/6 and NOD/Lt strains demonstrated the expected loss of PSP expression between 2 and 3 weeks of age (Fig. 5). Thus, re-expression of PSP in the submandibular gland in adult NOD mice exhibiting autoimmune exocrinopathy (19, 20) may represent derepression of an earlier developmental stage, and/or altered cell signals from the surrounding tissue microenvironment.

Expression of extracellular matrix and matrix degrading enzymes in salivary tissue

The retarded early development and loss of differentiated function of the salivary gland of NOD mice could be explained by an alteration in the extracellular signals used by

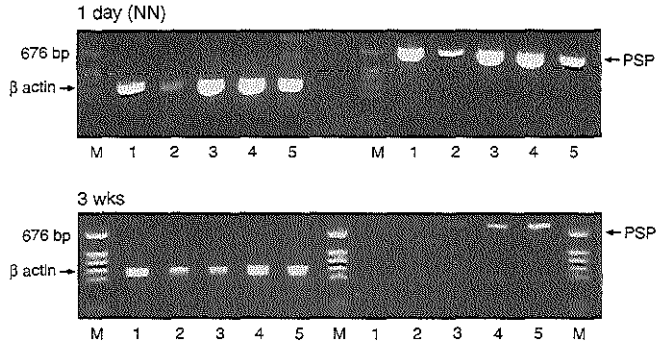

Figure 4
RT-PCR analysis of PSP mRNA expression in submandibular glands. Each reaction was performed on two separate occasions with mRNA prepared from the pooling of submandibular glands from a minimum of two litters of neonatal mice (78, 79). The housekeeping gene product β-actin was amplified as an internal control. As indicated, the top panel represents the amplicons generated with RNA isolated from 1-day-old mice, while the lower panel presents the amplicons generated from RNA isolated from the submandibular glands of 3-week-old mice. Lane 1, C57BL/6 mice; lane 2, NOD/Lt; lane 3, NOD.B6*Idd3*; lane 4, NOD.B10*Idd5*; lane 5, NOD.B6*Idd3*.B10*Idd5*. Molecular weights (M) are represented by a Promega 1 kb ladder.

cells to modulate proliferation and apoptosis (34-36). These typically are represented by cell-matrix interactions (7, 37). Cell attachment to the ECM activates growth promoting signaling pathways that are responsible for the anchorage requirement. The mRNA levels for two basement membrane ECM molecules identified as important contributors to exocrine tissue organogenesis appear to be normal (results not shown). An evaluation of MMP expression revealed high levels of MMP-9 mRNA in neonatal glands from CD1 and C57BL/6 mice. MMP-9 is one of the enzymes for which collagen IV is a substrate, (21, 38-40). Interestingly, gelatinase activities of MMP-2 and MMP-9 were reduced in NOD/Lt, NOD.B10.$H2^b$ and NOD.B10.$H2^b$-*scid* mice as compared to CD1 and C57BL/6 mice (Table 2). However, while the enzymatic activity declined in the control CD1 and C57BL/6 mice over the first 21 days of *postpartum* development, the gelatinase activity in the NOD congenic strains increased 2 to 3-fold (Table 2; $p < 0.05$).

Table 2. Evaluation of Type IV collagenase activity in salivary gland lysates

	CD1	NOD/Lt	NOD.B10.$H2^b$	NOD.B10.$H2^b$-*scid*
1 day	0.72 ± 0.43	0.37 ±0.24[a]	0.33 ± 0.12[a]	0.09 ± 0.02[a]
14 day	0.32 ± 0.15	0.86 ± 0.22[a]	0.70 ± 0.24[a]	ND
21 day	0.47 ± 0.19	2.61 ± 1.14	3.16 ± 0.63	ND

The MMP-2 and MMP-9 collagenase activity in neonatal submandibular glands was measured using a Chemicon (Temecula, CA) assay kit. A unit of activity is defined as 1.0 μg FITC-labeled type IV collagen substrate degraded/min/mg gland lysate, based on the fluorescence intensity using 520 nm (Em) and 495 nm (Ex). [a]Statistically significant differences, relative to the MMP-9 activity of CD1 control mice are reported as $p < 0.05$.

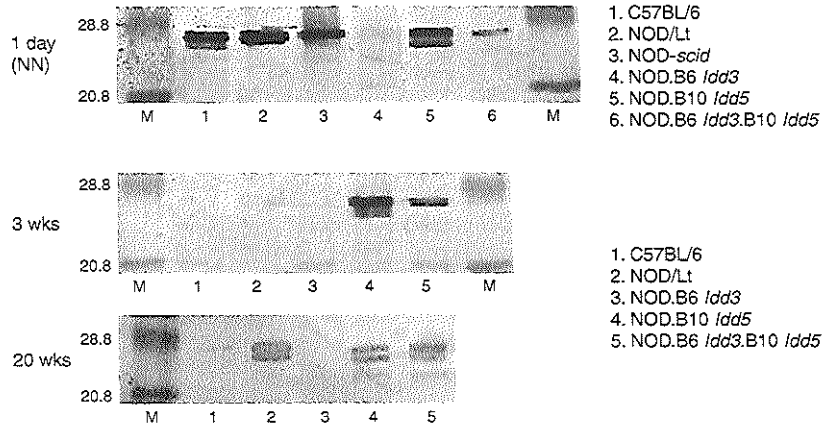

Figure 5
Western blot detection of PSP in submandibular gland lysates. The Western blot profiles for PSP expression in neonatal, 3-weeks, and 20-week-old mice (top, middle, and bottom panels, respectively) are presented as indicated in the figure. Molecular weights (M) are 28,000 Da, soy bean trypsin inhibitor; 20,000 Da, lysozyme (Bio-Rad).

Control of morphogenesis and developmental gene expression is mediated by alleles on chromosome 1 and 3.

Recently, evidence has been presented that autoimmune exocrinopathy is dependent on two chromosomal regions: *Idd3* and *Idd5* genetic regions on chromosomes 3 and 1, respectively (28). Replacement of *Idd3* and *Idd5* susceptibility alleles in NOD mice with the non-susceptibility alleles derived from non-autoimmune control C57BL mice, resulted in substantial changes in the glandular morphology during the neonatal phase (Fig. 1, D and E). Replacement of only the *Idd3* allele (NOD.B6*Idd3*) had the least impact on correcting the wide connective tissue septa and aberrant lobular organization of the acinar and ductal cells, while replacement of both *Idd3* and *Idd5* NOD alleles had the greatest impact. Replacement of *Idd5* alone showed an intermediate restoration of normal developmental patterns. As with the NOD/Lt and NOD.B10.$H2^b$ congenic strains, NOD.B6*Idd3*, NOD.B10*Idd5*, and NOD.B6*Idd3*.B10*Idd5* demonstrated normal expression of mRNA for EGF, collagen IV, integrins, TIMP-2, and fibronectin (results not shown).

Interestingly, expression of PSP was strikingly different between the congenic NOD strains. Replacement of the *Idd3* susceptibility allele in the NOD background showed normal steady state levels of PSP mRNA in the neonatal mice (Fig. 4). At 21 days, as well as in adult, very little if any protein was detected by Western blot (Fig. 5). In contrast, CD1, C57BL/6, and NOD/Lt mice had very little PSP mRNA detectable by 3 weeks of age, which correlated with the inability to detect the corresponding protein synthesis through Western blot analysis. On the other hand, the NOD.B10*Idd5* and NOD.B6*Idd3*. B10*Idd5* congenic

mice continued to express neonatal levels of PSP mRNA at 3 weeks of age as well as at the time of onset of autoimmune exocrinopathy at 20 weeks. The unique NOD-specific PSP isoform (19) was present in glandular protein profiles of NOD.B10*Idd5* at all three ages examined, whereas in the double congenic the normal isoform was synthesized at birth and 3 weeks of age, but was aberrantly processed at 20 weeks of age (Fig. 5).

***Idd3* and *Idd5* alleles alter gelatinase proteolytic levels in exocrine tissues.**

The C57BL resistance alleles replacing the NOD *Idd3* and *Idd5* susceptibility intervals were capable of altering the expression levels of gelatinase activity in neonatal and 21 day old submandibular glands. As presented in Figure 6, neonatal and 21-day-old NOD.B6*Idd3* mice had significantly elevated levels of gelatinase (MMP-2 and MMP-9) enzyme activity as compared to C57BL/6 control mice ($p < 0.05$ and $p < 0.01$, respectively). This level of gelatinase activity for NOD.B6*Idd3* was comparable to that observed in adult NOD/Lt mice with onset of autoimmune disease (28). In contrast, NOD.B10*Idd5* and NOD.B6*Idd3*.B10*Idd5* mice had patterns of gelatinase activity more consistent with the patterns of expression observed with healthy control mice than the parental NOD/Lt strain (Fig. 6).

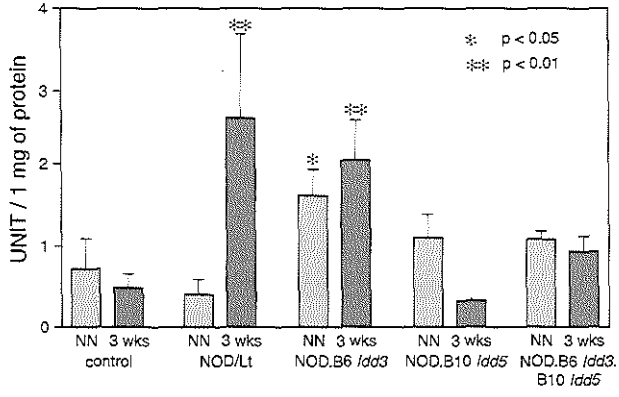

Figure 6
Histogram of gelatinase activity in submandibular glands prepared from NOD/Lt and congenic strains using neonatal (NN) and 3-week-old animals. Enzyme activity was determined as indicated in Table 2. *p<0.05; **p<0.01 vs. control mice.

Discussion

Altered ECM observed in autoimmune exocrine tissue pathology.

Studies on immunodeficient NOD-*scid* mice indicate abnormal glandular homeostasis in the absence of adaptive immune autoaggression and clinical symptoms (19, 20). To iden-

tify intrinsic factors that may trigger Sjögren's syndrome-like disease in the NOD genetic background mice, we compared neonatal submandibular gland cyto-and morpho-differentiation in the disease free state among strains less than 24 hours after birth using histomorphologic and biochemical analysis. Normal glandular morphogenesis in mice occurs during fetal development (day E11) when groups of cells of the primitive oral epithelium form focal clusters. On day E12, the primitive gland develops a club-like appearance and branching morphology, surrounded by a basement membrane. The full expression of the proper morphogenesis and cytodifferentiation appears to be modulated at this stage by the remodeling of the ECM through the expression of type I, type III, and type IV collagen, along with MMPs for ECM degradation (9, 11, 41). Unbalanced expression of those molecules results in abnormal gland development and loss of glandular homeostasis (11, 42), which is similar to observations in the glands of autoimmune disease-prone NOD mice.

Altered glandular homeostasis in human Sjögren's syndrome patients has also been reported. Biopsies from patients show significant increases in laminin protein and steady state concentrations of mRNA compared to normal control tissue (43). This suggests that altered basement membrane expression is an early event associated with salivary gland pathology in Sjögren's syndrome. Consistent with this hypothesis, elevated MMP-9 activity in both labial salivary glands and saliva in patients indicates increased remodeling and/or structural destruction of the basement membrane scaffolding in salivary glands (44, 45). Due to the role of basal lamina as an important molecular sieve and in extracellular matrix signaling, these pathological changes may contribute to the pathogenesis of the syndrome through altered cell homeostasis and induction of apoptosis (Fig. 7).

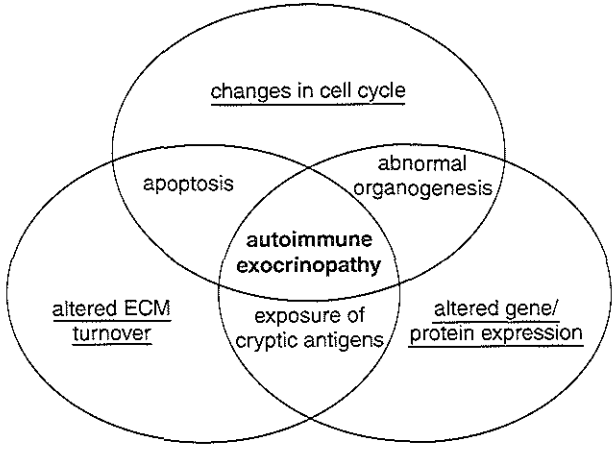

Figure 7
Schematic representation of the potential global factors influencing organogenesis and alterations generally leading to autoimmune disease.

Responses of cell proliferation to changing ECM signaling

The low levels of cell proliferation detected in the NOD neonatal submandibular glands by BrdU immunohistochemical detection is consistent with wide connective septa and less compact lobule structures apparent in the tissue section histology. The cell cycle machinery is composed of two core components, cyclin-dependent kinase (cdks) and cyclins. Extracellular physiological signals, such as growth factors, cell-matrix interactions, or cytokines, alter the expression of cyclins or cdk inhibitors, thus influencing the activity of cyclin:cdk complexes. Intracellular signals, such as integrity of the cell's own internal metabolism and its genome, are also important for the control of cell cycle, mediated by p53 and p21, which may inhibit the cell cycle or activate apoptosis (46). Whether the disturbance in cell proliferation in NOD mice is due to the intrinsic problems in the cell cycle unique to the NOD mouse genome, or in the exogenous signals affecting the decision of the cells to proliferate, differentiate, or undergo apoptosis needs to be further determined.

The observation that the earliest stages of organogenesis can be impaired in the offspring of women with diabetes suggest that abnormal metabolism disturbs embryogenesis. A mouse strain with elevated glucose demonstrated disrupted expression of genes regulating embryonic development and cell cycle progression, thereby causing premature cell death in emerging organ structures and defective morphogenesis (47). However, using NOD.B10.$H2^b$ congenic mice, which do not carry the predisposing diabetogenic MHC locus, and NOD-*scid* mice, which eliminates the possible differences in glucose level or in maternal diabetogenic IgG crossing over to the placenta, discount their influence on neonatal exocrinopathy.

Lower cell proliferation rate in the glands of NOD may link to altered MMP activity.

Lower than normal levels of MMP activity were detected in neonatal submandibular glands from NOD mice. Clearly, this reduced activity influences the rate of ECM remodeling taking place in both fetal and *postpartum* glandular development. This reduced activity may interfere with normal proliferation by creating a barrier around the cells (Fig. 7). Conversely, when cells are not proliferating efficiently, they may not up-regulate MMP activity to degrade ECM molecules for the proper signaling for proliferation, migration and differentiation. This may contribute to alterations in the morphology of neonatal glands, which in exocrine tissues results in less developed acinar lobules and wider connective septa compared to normal mice.

Decreased MMP activity in NOD neonatal submandibular glands may be due to decreased mRNA expression or stability, abnormal subcellular localization, or changes in TIMPs affecting protein expression and their activity, respectively. The increase in MMP activity at 3 weeks of age may be a consequence of ongoing abnormal organogenesis in the glands or to the inflammatory response of activated macrophage and dendritic cells in these tissues (48). Interestingly, the earliest infiltrating immune cells, the dendritic cells, accumulate in the submandibular glands of NOD and NOD-*scid* mice before the age of 5 weeks (49). These cells are capable of interfering with epithelial growth and differentiation (50). Increased matrix remodeling, indicated by an upregulation of MMP-9 enzymatic activity,

might lead to early glandular homeostatic dysregulation, thereby establishing the basis for autoimmunity within the submandibular gland.

Aberrant MMP-9 expression and activity has been described in both the NOD mouse and patients with Sjögren's syndrome (21, 41, 44, 45). Elevated MMP activity has been proposed to occur in the diseased tissue in association with epithelial cell replacement and maintenance of the acinar cell component of the exocrine tissues (21). Activation of the apoptotic signaling cascade may result from induced changes in cell shape generated by MMP alterations of ECM composition. The increased MMP activity therefore may be a cellular response to re-establish the surrounding microenvironment to resume epithelial cell differentiated function and reverse apoptosis or encourage proliferative activity of the tissue (Fig. 7). Clearly, further evaluation of embryonic organogenesis may provide evidence for this possibility. Increased MMP activity may be related to disease progression or release of cell surface receptors and cytokines, such as TNF-α or ECM-associated growth factors (51-55). The induction of MMP-9 mRNA and proteolytic activity observed in a human salivary cell line and in the saliva of Sjögren's syndrome patients has been proposed to arise as a consequence of the presence of IFN-γ (56) or TGF-α, a cytokine involved in immunoregulation, embryonic development and wound healing (5).

Loss of attachment to the ECM causes apoptosis in many cell types (37). The surface of apoptotic cells exhibit membrane blebs that contain potential autoantigens (22). Elevated Fas and Fas ligand expression detected in the gland of NOD mice at 3 days of age by immunohistochemistry may indicate alterations in normal growth and differentiation of the glands. The absence of significant differences between control and NOD mice in DNA fragmentation detected by TUNEL staining, may indicate that there is a balance between regulatory mechanisms, such as elevated bcl-2 expression, in the NOD gland that prevent cells from undergoing irreversible cell death. Additionally, integrins (58) appear to play a major role in conveying survival signals from the ECM by inactivating two pro-apoptotic proteins, Bad and caspase-9, via focal adhesion kinase (FAK) activation. Integrins also regulate genes that are important for cell proliferation by induction of the AP1 transcription factor. In our study, there were no significant differences in the steady state of integrin α_v mRNA levels, a protein present in the basal lamina of ductal and acinar cells of the glands (59).

Genetic control of submandibular gland morphogenesis and autoimmune exocrinopathy

Genes within the chromosome regions *Idd3* and *Idd5*, appear to have an additive influence on adult onset of Sjögren's syndrome-like pathophysiology in the NOD background (28). Backcross of *Idd5* derived from NOD onto the genome of normal C57BL/6 mice renders an almost full expression of disease phenotype at 20 weeks of age. Replacement of chromosomal intervals for *Idd3* and *Idd5* derived from normal mice appear to lead to protection from autoimmune disease. These included a less severe tissue pathology as reflected in reduced caspase and gelatinase activity in the exocrine tissues. Similarly, these genetic intervals had a hierarchical effect on submandibular gland morphogenesis.

Our neonatal study also supports the hypothesis that non-immune genetic components may play an important role in the disease pathogenesis. The *Idd3*-derived interval from C57BL/6 appears to alter PSP protein expression patterns in the NOD background. However, submandibular gland organogenesis as well as the biochemical and physiological pathology associated with Sjögren's syndrome-like disease of adult NOD mice was least influenced by this region. Mapping of PSP to the *Idd13* region on chromosome 2 suggests that this gene is not one of the primary autoantigens initiating autoimmunity, but its expression may be regulated by transacting regulators on chromosome 3. In contrast, in congenic NOD mice with the *Idd5* region derived from C57BL/10, PSP expression was not downregulated, and additionally showed a proteolytic cleavage pattern throughout glandular development. MMP activity in the NOD.B10*Idd5* mice was downregulated similar to expression patterns observed with normal healthy mice rather than the parental NOD/Lt mice.

Morphological analysis of submandibular glands from the congenic mice with *Idd3* and *Idd5* showed the similar gross morphology and almost normal lobular pattern of control mice, although lobular structures were slightly more fragmented than in the controls suggesting the importance of these two loci in autoimmune exocrinopathy and the possible contributions of other genes on other chromosomes. Genes in the *Idd5* region, such as fibronectin 1, pro-collagen type IV alpha 3, cadherin 7, plasminogen activator inhibitor type II, microtubule associated protein 2, ribosomal proteins, insulin-like growth factor binding protein 2 and 5, and cathepsin E, may play a role in abnormal organogenesis and subsequent generation of autoantigens that set the stage for autoimmune exocrinopathy. Recent studies suggest that a number of ribosomal proteins have secondary functions, such as cell proliferation regulators and in some instances as inducers of cell death, independent of their involvement in protein biosynthesis (37). Insulin-like growth factor binding proteins (IGFBPs) may also play a role in affecting cell growth. The addition of IFN-γ and TNF-α in combination with IGF-I to the salivary cell line HSG, enhanced the expression of IGFBP-3, -4, and –5, resulting in increased cell growth (60).

Development of an autoimmune response no doubt relies on intrinsic and extrinsic cellular events. Recent observations suggest that target organs are not merely passive targets of autoimmunity, but intimate participants in the initiation of the pathogenesis (19, 20). From the present studies, it is clear that, a potential link between tissue targeting by the immune system and a specific aberrant development during the fetal and/or neonatal period has been identified. This is not to say that the abnormal development, which ultimately affects the physiological and biochemical events within these tissues, is the cause, but the correlation is very interesting. The NOD mouse is well known to have several immunological defects, including delayed maturation of the monocyte, C5-deficiency, and low NK cell activity. All of these defects may contribute to the development and/or maintenance of autoimmunity in this mouse strain. Furthermore, as outlined in Figure 7, organogenesis, as well as tissue health, is dependent on the interactions of the ECM, cellular proliferation rates and protein expressions. A shift in the homeostasis of these factors during organogenesis could easily promote the onset of an immunological attack.

Acknowledgments

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Chapter 6

CHEMOKINE EXPRESSION DURING THE DEVELOPMENT OF SIALOADENITIS IN THE NOD MOUSE MODEL FOR SJÖGREN'S SYNDROME

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Abstract

We have previously demonstrated that the development of sialoadenitis in the nonobese diabetic (NOD) mouse, which is a model for Sjögren's syndrome, is preceded by an influx of dendritic cells into the submandibular glands (SMG). As dendritic cells were not detected in SMG of control mice, we proposed an important role for these antigen presenting cells in the initiation of the autoimmune reaction. The signal, responsible for the accumulation of dendritic cells is not known. As chemokines can influence the traffic of leukocytes, we examined if they could be responsible for the attraction of dendritic cells into the NOD SMG. Therefore, chemokine mRNA expression was studied in SMG of NOD, NOD-*scid* and control mice by RNAse protection assay (RPA). This revealed the expression of MIP-1α, MCP-1, RANTES, and eotaxin in SMG of these mouse strains from 3 through 18 weeks of age. Furthermore, induced expression of IP-10 in SMG of NOD mice aged 12 weeks and older was revealed. The protein and mRNA expression of MIP-1α, MCP-1, and RANTES in SMG were also quantitatively analyzed. No differences were observed between the mouse strains early in the disease process. Following the onset of sialoadenitis in NOD mice, expression of MIP-1α, MCP-1, and RANTES was increased. We conclude that the influx of dendritic cells into the NOD SMG, before the onset of sialoadenitis, is not due to altered expression levels of MIP-1α, MCP-1, or RANTES. The alterations in chemokine expression may indicate, however, that these chemokines do play a role in the exacerbation of the autoimmune response, following the development of lymphocytic infiltrates.

Introduction

The nonobese diabetic (NOD) mouse strain is widely used as a model for Sjögren's syndrome, an autoimmune exocrinopathy that is characterized by the development of lymphocytic infiltrates in the salivary and lacrimal glands (1). Concurrent with lymphocytic infiltration of these glands, a decreased secretory response is observed in NOD mice and in part of the patients. In patients, this ultimately results in disease manifestations as dry eyes and a dry mouth (2). Although the decreased production of saliva and tears has long been thought to result from cytotoxicity excerted by cells of the lymphocytic infiltrates, either via the induction of apoptosis or via the release of cytotoxic mediators, evidence for an autoantibody dependent mechanism in this phase of the disease is now accumulating (3, 4). While the insight in processes involved in the late phase of the autoimmune process is increasing, still little is known about the events that play a role in its initiation. Mouse models for Sjögren's syndrome enable studying this early phase of the autoimmune disease.

We have previously shown that the development of lymphocytic infiltrates in the submandibular glands (SMG) of NOD mice (sialoadenitis), observed from the age of 10 weeks onwards, is preceded by an influx of dendritic cells into these glands, occurring between 2

and 5 weeks of age (5). This influx occurred in part independent of the presence of lymphocytes (6), since it was also observed in NOD-*scid* mice, although to a lesser extent. In the MRL/lpr mouse, another model for Sjögren's syndrome, an accumulation of dendritic cells into the SMG did not occur before the development of sialoadenitis. As dendritic cells are unique in their ability to prime naïve T lymphocytes and to initiate an immune response (7, 8), an important role for these cells in the initiation of sialoadenitis in the NOD mouse can be envisaged .

The question evolving from these observations is, why do dendritic cells accumulate in the SMG of NOD and NOD-*scid* mice? The cause of the accumulation may reside in the dendritic cell population, in the SMG, or both. Abnormal expression of chemoattractants by glandular cells could result in the attraction of dendritic cells. In addition, a disturbed responsiveness of NOD dendritic cells to signals, normally produced within the SMG, may play a role.

Chemokines are molecules that can tightly regulate the traffic of dendritic cells. Immature dendritic cells express receptors that bind several inducible chemokines, such as macrophage inflammatory proteins (MIP), monocyte chemotactic proteins (MCP) and RANTES. Inducible chemokines are generally expressed at sites of inflammation, in contrast to constitutive chemokines, which are mainly expressed in secondary lymphoid organs. Upon maturation of dendritic cells, induced by antigen uptake or by exposure to inflammatory signals, the receptors for inducible chemokines are downregulated, which is accompanied by an increased expression of receptors for constitutive chemokines (9, 10). This change in chemokine receptor expression pattern allows dendritic cells to leave the site of inflammation and to migrate towards the secondary lymphoid organs, where they may activate antigen-specific lymphocytes.

In this study, we examined the expression of chemokines, known to be chemotactic for immature dendritic cells, in submandibular glands of NOD and NOD-*scid* mice of various ages. This was done at the mRNA (RNAse protection assay; RQ-PCR) as well as the protein level. The reason for using NOD-*scid* mice in addition to the NOD mice is that the former lack functional B and T lymphocytes (6). C57BL/10 and BALB/c mice were used as control strains. The chemokine expression at the various ages studied was carefully compared to the appearance and accumulation of dendritic cells and to the development of lymphocytic infiltrates and the onset of sialoadenitis. As the expression of chemokines can be induced by proinflammatory cytokines (11-15), we also investigated the mRNA expression of IL-1ß, IL-6, and TNF-α in SMG of the different mouse strains at the various ages.

Materials and methods

Mice and experimental design

Female NOD, NOD-*scid*, and C57BL/10 mice were bred in our own facilities under specific pathogen-free conditions. Mice were fed standard pellets and water *ad libitum* and

were maintained at 22°C +/- 1°C on a 12-hour light/ 12-hour dark cycle. Under these conditions, the incidence of diabetes in NOD mice at 30 weeks of age was 90% in females and 30% in males. Female BALB/c mice were purchased from Harlan (Horst, The Netherlands) and housed under the same conditions. Mice, aged 3 weeks and older, were killed by asphyxiation with carbon dioxide. Mice, younger than 3 weeks were killed by cervical dislocation.

RNA isolation

SMG were removed, homogenized in RNAzol^{TM} B (Campro Scientific, Veenendaal, The Netherlands), and stored at -80°C until further processing. Total RNA was extracted from these homogenates according to the manufacturers protocol. The yield and purity of the RNA was determined by measuring the OD260 and OD280. Samples were prepared by pooling the RNA of three mice. Of each mouse strain, two pooled samples were prepared per age group, which were used in consecutive experiments.

RNAse protection assay

A multi-probe template set (mCK-5, containing DNA templates for Ltn, RANTES, Eotaxin, MIP-1ß, MIP-1α, MIP-2, IP-10, MCP-1, TCA-3, L32, GAPDH) was purchased from Pharmingen (San Diego, CA). This template set was used to synthesize the [α^{32}P]UTP (3000 Ci/mmol, 10 mCi/ml, Amersham Life Science, Amersham, GB) labeled probes in the presence of a GACU pool using a T7 RNA-polymerase, according to the manufacturers protocol. For generation of the probes and for the subsequent RNAse protection assay (RPA) procedure, an *in vitro* transcription kit and an RPA kit were used (Pharmingen). For each sample, 5 µg of target RNA was hybridized with the labeled probes overnight, which was followed by digestion with RNAse A and T1. Subsequently, the samples were treated with proteinase K, which was followed by phenol/ chloroform extraction and precipitation in the presence of ammonium acetate. The samples were loaded on an acrylamide/ urea sequencing gel next to the labeled, undigested probe, and run at 50W under 0.5 × TBE. The gel was dried under vacuum and exposed on Kodak X-AR film with intensifying screens at -70°C.

cDNA synthesis

Target RNA (1 µg) was reverse transcribed using per reaction: 5U AMV-RTase, 2 µl 10× concentrated AMV RT buffer, 1 µl 20 mM dNTP mix, 2 µl 10 mM spermine/HCl, 1 µl 40 U/µl RNA guard, 0.5 µl 100 OD/ml random hexamers, and 2 µl 100 µg/ml oligo(dT)$_{15}$. This reaction mixture was adjusted with H_2O to a total volume of 20 µl, incubated at 41°C for 1 hour and stored at -80°C.

Primers and probes

PCR primers and fluorogenic probes for the target genes MCP-1, IL-1ß, and GAPDH were designed using the computer program Primer Express, and were purchased from PE Biosystems (Branchburg, NJ). The oligonucleotide sequences of the primers, used for the

detection of expression of these genes are: MCP-1 3', AGT AGG CTG GAG AGC TAC AAG AGG; MCP-1 5', TTG AGC TTG GTG ACA AAA ACT ACA G; IL-1ß 3', CAA CCA ACA AGT GAT ATT CTC CAT G; IL-1ß 5', GAT CCA CAC TCT CCA GCT GCA; GAPDH 3', TTC ACC ACC ATG GAG AAG GC; GAPDH 5', GGC ATG GAC TGT GGT CAT GA. The oligonucleotide sequences of the fluorogenic probes are as follows: MCP-1, CAC CAG CAG CAG GTG TCC CAA AGA A; IL-1ß, CTG TGT AAT GAA AGA CGG CAC ACC CAC C; GAPDH, TGC ATC CTG CAC CAC CAA CTG CTT AG. The fluorogenic probes contained a reporter dye (FAM) covalently attached to the 5' end and a quencher dye (TAMRA) covalently attached to the 3' end. Extension from the 3' end was blocked by attachment of a 3' phosphate group. For the detection of TNF-α, IL-6, MIP-1α, and RANTES mRNA expression, Pre-Developed Taqman® Assay Reagent kits were purchased from PE Biosystems (Branchburg, NJ).

PCR amplification

PCR reactions were performed in the ABI-prism 7700 sequence detector, which contains a Gene-Amp PCR system 9600 (Perkin Elmer/ Applied Biosystems, Foster City, CA). Reaction conditions were programmed on a Power Macintosh 7200, linked to the sequence detector. PCR amplifications were performed in a total volume of 25 µl, containing 2 µl cDNA sample (1 µl for GAPDH), 12.5 µl 2× concentrated Taqman® Universal PCR Master Mix (PE Biosystems), and 900 nM of each primer (for MCP-1 and GAPDH detection). Each reaction also contained 200 nM of the corresponding detection probe (for MCP-1 and GAPDH detection). For the detection of IL-1ß expression, 1200 nM of each primer and 250 nM of the detection probe were used. Primers and probes used for the detection of TNF-α, IL-6, MIP-1α, and RANTES were diluted 20 times, according to the manufactures protocol. PCR amplification reactions were performed in duplicate wells, using the following conditions: 2 min at 50°C and 10 min at 95°C, followed by a total of 50 two-temperature cycles (15 s at 95°C and 1 min at 60°C).

ELISA

Salivary gland lysates were prepared by homogenization of SMG in ice-cold Hank's buffer (Life Technologies, Paisley, United Kingdom) supplemented with Protease Inhibitor Cocktail (Boehringer Mannheim, Mannheim, Germany), 1 tablet in 10 ml Hank's buffer. Subsequently, salivary gland lysates (n = 4 to 11 mice/ age/ strain), were sonicated twice for 30 seconds and centrifuged at 10000g, 4°C, for 10 minutes, after which the supernatant was carefully removed. Protein concentration in the supernatant was determined using the Bio-rad protein assay (Bio-rad laboratories, GmbH, München, Germany). Quantikine murine MIP-1α and MCP-1 ELISA kits were purchased from R&D systems (Minneapolis, MN), and used, according to the protocol, supplied by the manufacturer.

Statistical analysis

Protein levels, measured in submandibular glands were averaged per age group, per

mouse strain. The differences between the means were evaluated by means of the Student's t-test. A p-value < 0.05 was considered statistically significant.

Results

Chemokine mRNA expression by RNAse protection assay

RNA from submandibular glands of NOD, NOD-*scid*, and BALB/c mice aged 3, 7, 12, and 18 weeks, was subjected to RNAse protection assay to study chemokine mRNA expression. Two consecutive experiments were performed, and similar results were obtained in both experiments. Clear expression of MIP-1α and eotaxin was detected in all mouse

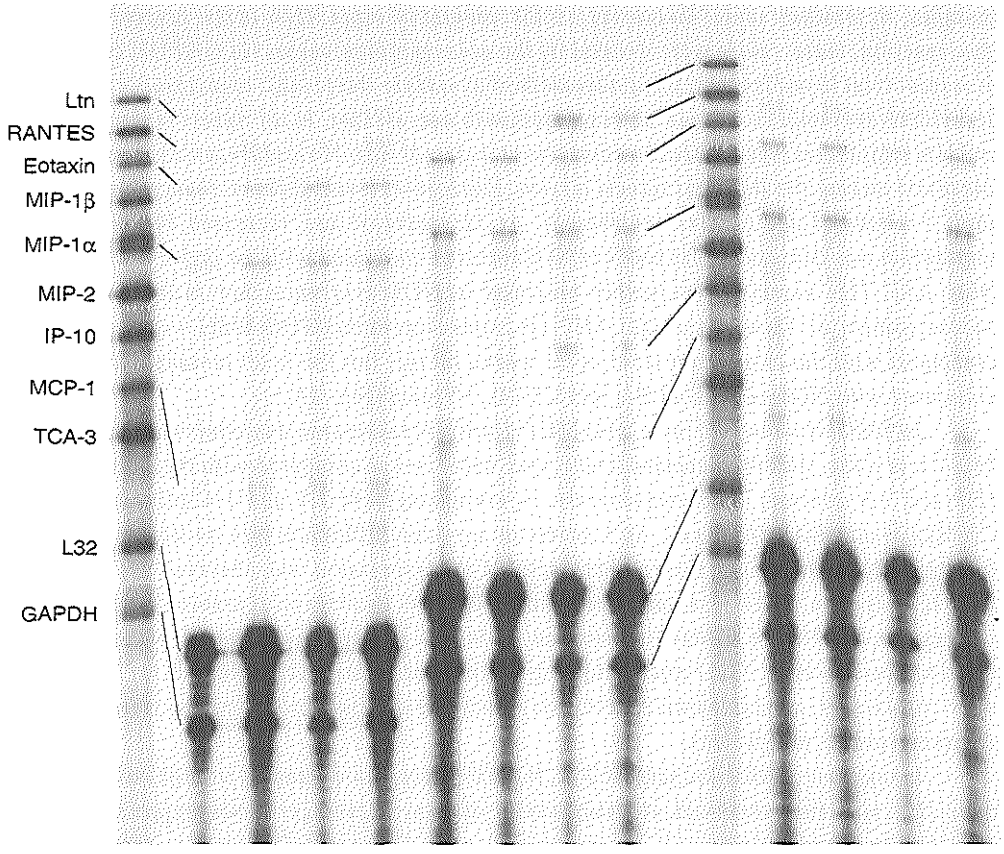

Figure 1
Autoradiograph of RNAse protection assay (RPA) on RNA, isolated from the submandibular gland of BALB/c, NOD, and NOD-*scid* mice. Below the lanes, the age of the mice is given in weeks. An undigested probe (P) was included to identify the protected bands in the samples. The chemokines examined with RPA included lymphotactin (Ltn), RANTES, eotaxin, MIP-1ß, MIP-1α, MIP-2, IP-10, MCP-1, and TCA-3.

strains, in all age groups (Fig. 1). Other chemokines that were detected in the majority of samples included RANTES and MCP-1. Expression of RANTES was increased in SMG of 12 and 18-week-old NOD mice, but not in NOD-*scid* mice. Furthermore, in glands of 12 and 18-week-old NOD mice, expression of IP-10 was revealed, which was absent in all other samples (Fig. 1). Messenger RNA samples of C57BL/10 mice were also examined for chemokine expression, and similar results were obtained as with BALB/c mice (results not shown).

Quantification of MIP-1α, MCP-1, and RANTES mRNA expression by RQ-PCR

To quantitate mRNA expression of MIP-1α, MCP-1, and RANTES in SMG, RQ-PCR reactions were performed. In each experiment, a standard curve was generated in which the threshold cycle was plotted against the starting quantity of input cDNA. This curve was used to calculate the starting quantity of mRNA expression of the individual chemokines in the samples, in arbitrary units. To correct for the amount of input RNA in the reverse transcriptase reaction, these values were divided by the corresponding values for GAPDH expression. It is important to realize that, using this method to analyse the results, expression levels of the individual chemokines can not be compared with one another. In all mouse strains, MIP-1α mRNA expression was detected as early as at 1 day of age. No significant differences were observed among the different mouse strains. Expression levels did not change significantly until 7 weeks of age (results not shown). At 12 weeks of age, increased MIP-1α mRNA expression was detected in the SMG of NOD mice as compared with the previous time points (Fig. 2A). This increase was neither observed in NOD-*scid* mice, nor in the two control mouse strains. At 18 weeks of age, MIP-1α expression in the NOD SMG was decreased as compared with the expression level at 12 weeks of age, but the level remained elevated as compared with NOD-*scid*, BALB/c and C57BL/10 mice (Fig. 2A).

In SMG of 1-day-old mice, also MCP-1 mRNA expression was observed. In NOD and NOD-*scid* mice, slightly increased levels were measured as compared with the control strains (2.5 and 1.6 arbitrary units respectively, as compared with 1.1 and 1.4 arbitrary units in C57BL/10 and BALB/c mice). At 3 weeks of age, this difference was no longer observed. Interestingly, in all mouse strains decreased MCP-1 expression was observed at 7 weeks of age (Fig. 2B). In NOD-*scid* and control mice, MCP-1 expression remained relatively low whereas in NOD mice it was increased at 12 weeks of age. At 18 weeks of age, MCP-1 mRNA expression in NOD mice returned to the expression level observed in 7-week-old mice. At this age, no significant differences among the different mouse strains were observed anymore (results not shown).

In contrast to MIP-1α and MCP-1, RANTES was not expressed in SMG of 1-day-old mice, but was first observed at three weeks of age. Messenger RNA expression levels remained constant in time in all mouse strains, except for the NOD mouse, in which an elevated expression was observed at 12 weeks of age (Fig. 2C). In SMG of 18-week-old NOD mice, RANTES expression was decreased as compared with 12 weeks of age. The expression levels among the different mouse strains were similar at that time (results not shown).

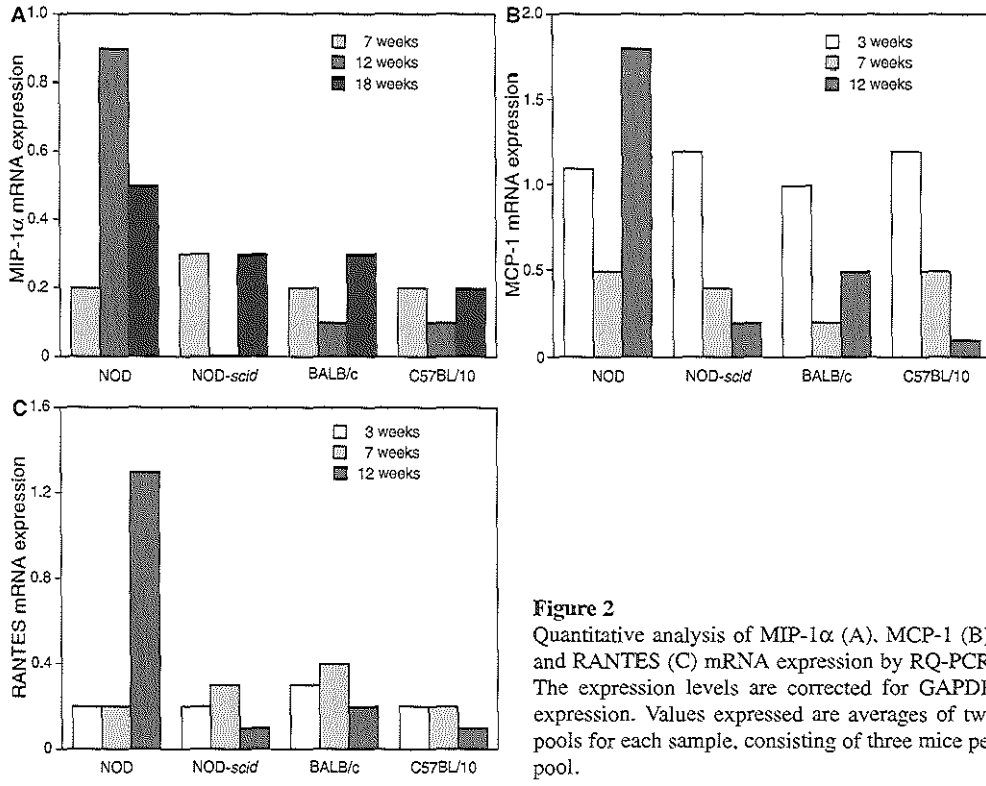

Figure 2
Quantitative analysis of MIP-1α (A), MCP-1 (B), and RANTES (C) mRNA expression by RQ-PCR. The expression levels are corrected for GAPDH expression. Values expressed are averages of two pools for each sample, consisting of three mice per pool.

Protein expression of MIP-1α and MCP-1 in submandibular glands

To examine whether mRNA expression of the chemokines MIP-1α and MCP-1 was accompanied by their protein expression, and to quantify this expression, ELISA were performed on SMG lysates of mice, from 3 through 18 weeks of age. MIP-1α protein was detected in SMG of all mouse strains, and at all ages tested. Although at 3 and 7 weeks of age MIP-1α levels were similar among the different mouse strains, significantly increased levels were measured in NOD mice at 12 and 18 weeks of age, as compared with age-matched control mice (Fig. 3A). When compared with NOD-*scid* mice, a significant increase was only observed at 18 weeks. Interestingly, when the expression levels in individual mice were examined, it was found that within the group of 12-week-old NOD mice two subgroups could be distinguished, based on the MIP-1α levels that were measured in SMG lysates of these mice. In one subgroup (7 of 11 mice), expression levels ranging from 101-194 pg MIP-1α/100 mg protein were measured, whereas levels measured in lysates of the other 4 mice ranged from 426-639 pg MIP-1α/100 mg protein. A similar distinction could be made among 18-week-old NOD mice, although the differences were attenuated as compared with 12-week-old NOD mice. In Fig. 3A, MIP-1α levels are expressed per 100 mg total protein. When the levels are expressed per 100 mg glandular tissue, to control for possible interfer-

ence of vascular protein leakage in SMG in which lymphocytic infiltrates had developed, a similar pattern was observed (results not shown).

MCP-1 protein was also detected in SMG of all mouse strains, from 3 through 18 weeks of age (Fig. 3B). In NOD and NOD-*scid* mice, but not in the control mice, a marked decrease in MCP-1 was observed at 7 weeks of age as compared with the 3 weeks time point. This decrease was statistically significant in both mouse strains ($p = 0.003$ and $p = 0.004$ in NOD and NOD-*scid*, respectively). In NOD mice, this decrease was followed by increased expression at 12 weeks of age, which was significant as compared to the 7 weeks time point. This increase was not observed in NOD-*scid* mice (Fig. 3B). At 18 weeks of age, the MCP-1 levels in NOD mice were significantly increased as compared with age-matched NOD-*scid* mice, but not when compared with control mice.

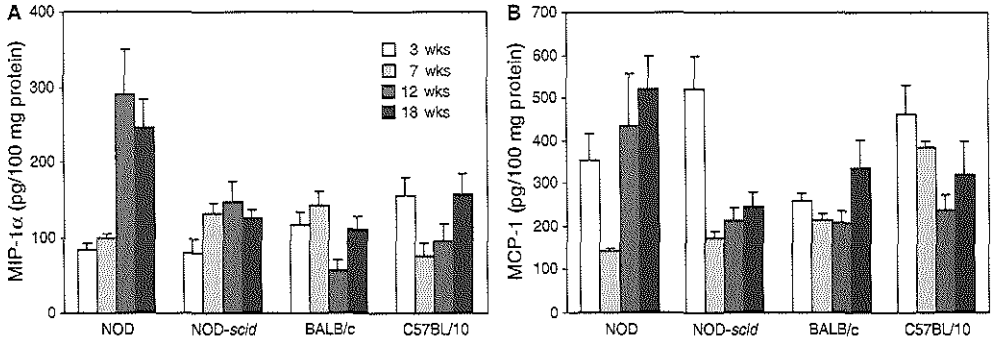

Figure 3
MIP-1α (A) and MCP-1 (B) protein expression in submandibular glands of 3 through 18-week-old NOD, NOD-*scid*, and control mice. Values are expressed as averages of 4-11 mice per mouse strain and per time point, +/- SEM.

Quantitative analysis of IL-1ß, IL-6 and TNF-α mRNA expression

Messenger RNA expression levels of the proinflammatory cytokines IL-1ß, IL-6 and TNF-α in SMG of the various mouse strains were examined by RQ-PCR. Expression of IL-1ß and TNF-α was detected from 1 day through 18 weeks of age in all mouse strains. No major differences were observed among the different mouse strains, and the expression remained at a similar level when followed in time (results not shown).

Interleukin-6 mRNA expression was also detected in SMG from 1 day of age onwards. At 1 day and three weeks of age, the levels measured in the different mouse strains were similar (Fig. 4). At 7 weeks of age decreased expression was observed in the NOD and control mouse strains, but not in NOD-*scid* mice. An increase was observed in 12-week-old NOD mice, which was not observed in the other mouse strains (Fig. 4). This expression level was still present in the NOD mouse at 18 weeks of age (data not shown).

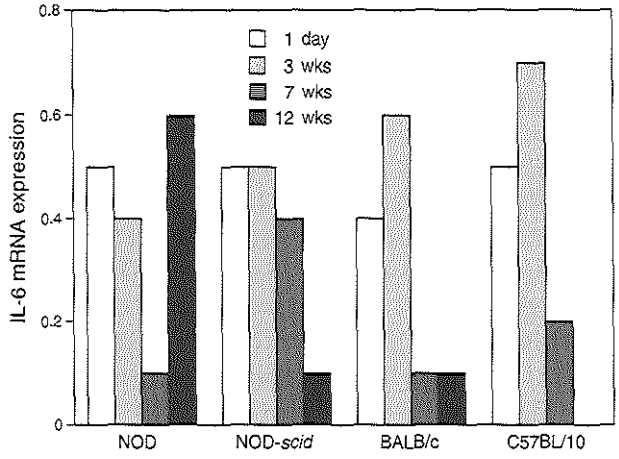

Figure 4
Qantitative analysis of IL-6 mRNA expression by RQ-PCR. The expression levels are corrected for GAPDH expression. Values expressed are averages of two pools for each sample, consisting of three mice per pool.

Discussion

In submandibular glands of NOD mice (but not of control BALB/c and C57BL/10 mice), an influx of dendritic cells occurs before the development of a lymphocytic infiltrate, suggesting that these cells pave the path for the sialoadenitis in the NOD mouse. The reason for this accumulation of dendritic cells is not known. Since immature dendritic cells express a specific combination of chemokine receptors, enabling these cells to respond to inducible chemokines, altered expression of these chemokines in the SMG of the NOD mouse may be responsible for the observed dendritic cell influx. By RNAse protection assay, we demonstrated mRNA expression of the chemokines MIP-1α, eotaxin, MCP-1 and RANTES in SMG of all mouse strains investigated, as early as three weeks of age, the earliest time point included in this experiment (Fig. 1). Expression of MIP-1α, MCP-1, and RANTES was further analyzed by RQ-PCR, and no significant differences in mRNA expression were found between the different mouse strains until 7 weeks of age (Fig. 2). Furthermore, no major differences in protein expression of MIP-1α and MCP-1 were found until 7 weeks of age (Fig. 3). Therefore we conclude that the influx of dendritic cells, occurring in SMG of NOD and NOD-*scid* mice between 2 and 5 weeks of age (5), is not due to an altered expression level of the chemokines MIP-1α and MCP-1. However, since abnormalities may exist in the expression and function of chemokine receptors on NOD dendritic cells, this does not exclude a role for these chemokines in the accumulation of dendritic cells in the NOD SMG.

Recently, hyperactivation of NF-κB in response to various forms of stimulation has

been described in dendritic cells of NOD mice (16). This defect was shown to result in increased expression of IL-12 by dendritic cells following stimulation, which was suggested to provide a microenvironment in which Th1 responses are favoured. Similarly, hyperactivation of NF-κB might, directly or indirectly, affect the expression of chemokine receptors.

Expression of MIP-1α, MCP-1, eotaxin and RANTES in SMG from an early age on was not specific for NOD and NOD-*scid* mice, but was also evident in the control mouse strains. It is possible that MIP-1α, MCP-1, and RANTES attract the resident macrophages, that we have observed in SMG of all mouse strains (unpublished observations). Eotaxin can bind to the CCR3 receptor expressed on eosinophils, basophils, mast cells and Th2 cells and can thereby attract these cell types (17-20). Although eosinophils and Th2 cells have not been described in non-diseased submandibular glands, we did observe significant numbers of mast cells in SMG of NOD, NOD-*scid* and control mice (unpublished observations).

It is remarkable that the expression of the chemokines MIP-1α, eotaxin, MCP-1, and RANTES is not accompanied by the development of an inflammatory infiltrate in glands of control mice. This suggests that chemokines may also serve another role than the attraction and activation of inflammatory leukocytes. Recently, it has become evident that several chemokines (including MCP-1, MIP-1α, RANTES and eotaxin) play a role in the regulation of angiogenesis and extracellular matrix (ECM) deposition (21-26). As these are essential processes in SMG development in the embryonic and neonatal stage of SMG development, as well as in maintenance of glandular homeostasis in the adult, a role for chemokines in these processes can be envisaged. It is also of interest to note that, in contrast to MIP-1α and MCP-1, RANTES mRNA expression was not observed in SMG of 1-day-old mice (Fig. 2). This suggests that RANTES does not contribute to SMG organogenesis in the developing embryo, but may influence glandular homeostasis later in life.

Although no differences were observed in chemokine expression among the different mouse strains early in life, we did note a significantly increased protein expression of MIP-1α and MCP-1 in NOD SMG from 12 weeks of age onwards (Fig. 3). Furthermore, mRNA expression of IP-10 was evident in 12 and 18-week-old NOD mice, while MIP-1α, MCP-1, and RANTES mRNA expression were increased at 12 weeks of age (Figs. 1 and 2). This may influence the composition of the lymphocytic infiltrates, as the receptors for these chemokines (CCR1, CCR2, CCR5, and CXCR3) are expressed on Th1 cells (27). Indeed, lymphocytes accumulating in NOD SMG during the development of sialoadenitis have been described to exhibit the Th1 phenotype (28-30).

The alterations in MIP-1α, MCP-1, RANTES, and IP-10 expression in the NOD mouse were due to the lymphocytic infiltrates, as they were not observed in NOD-*scid* SMG. The increased chemokine expression may be due to the leukocytes, present in the inflammatory infiltrates or in the glandular parenchyma, or to glandular epithelial cells, exposed to inflammatory mediators.

RANTES has recently been shown to induce the expression of a variety of chemokines and cytokines in murine bone-marrow derived dendritic cells including MIP-1α, MIP-1ß, MIP-2, RANTES, IL-6, and TNF-α (31). This indicates that dendritic cells, present in SMG

of NOD mice may as well be responsible for increased chemokine expression following exposure to proinflammatory cytokines or chemokines.

The development of sialoadenitis in the NOD mouse has been shown to coincide with increased expression of a wide array of cytokines, including IL-1ß, TNF-α, and IFN-γ (28-30). These cytokines can induce expression of MCP-1, MIP-1α, RANTES, and IP-10 in a variety of cell types, indicating that they may also contribute to alterations in chemokine expression observed in the SMG of NOD mice (14, 15, 32-38).

In summary, once lymphocytic infiltrates have started to develop in NOD SMG, chemokines and cytokines are produced that may induce an amplification cascade, in which lymphocytes, dendritic cells, and epithelial cells are involved, resulting in aggravation of the autoimmune response. This would be consistent with studies of the minor salivary glands of patients with Sjögren's syndrome, showing expression of MIP-1ß, MIP-1α, and RANTES in the infiltrating lymphocytes and in glandular epithelial cells. Although the exact role of chemokines in the development of Sjögren's syndrome remains to be established, their identification in the salivary glands of patients as well as in mouse models for Sjögren's syndrome are suggestive for a role in the disease process.

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Chapter 7

GENERAL DISCUSSION

Sjögren's syndrome is an autoimmune disease in which a chronic inflammatory process in a.o. the salivary and lacrimal glands is accompanied by dryness of the mouth and eyes (1, 2). Knowledge of the mechanisms, thought to be responsible for the decreased production of saliva and tears in Sjögren's syndrome has increased over the past years. Mechanisms which have been postulated to be responsible for the induction of death of glandular epithelial cells and the decreased secretory response include apoptosis and the release of cytotoxic mediators by lymphocytes present in the infiltrates (3-8). However, evidence has been put forward indicating that the decreased secretion does not correlate with the degree of lymphocytic infiltration or glandular damage in the minor salivary glands (9, 10). Recent studies suggest an important role for autoantibodies, directed towards the M3 muscarinic acetylcholine receptor in the decreased secretory response (11-14).

Studies on the initiation of Sjögren's syndrome have mainly focussed on the potential abnormalities in the adaptive component of the immune system, which may lead to the recognition of normal components of the exocrine glands and the induction of an autoimmune response (15-19). However, it may well be that in patients with Sjögren's syndrome glandular components, such as the epithelial cells, exhibit altered features, and are recognized as 'non-self'. Furthermore, glandular epithelial cells may produce mediators that create a proinflammatory environment in which an (auto)immune response is prone to develop. This may occur in the presence or absence of additional immune defects. The same may hold for other resident cells within the gland, such as fibroblasts or macrophages. In the studies described in this thesis, we investigated whether aberrances exist in the salivary glands which may contribute to the initiation of the sialoadenitis. If such aberrances do occur, this would suggest that the exocrine glands are not just passive targets of the autoimmune response, but instead could be active participants in the pathogenesis of sialoadenitis.

Most of the studies described in this thesis were performed in the submandibular gland (SMG) of the nonobese diabetic (NOD) mouse, as this enabled detailed analysis of the preautoimmune salivary gland. In this chapter, the results of the previous sections are discussed and, when applicable, extrapolated to the human situation. Furthermore, suggestions for future experiments are given.

Influx of dendritic cells prior to the development of sialoadenitis in the NOD mouse: role in the initiation of sialoadenitis?

The development of autoimmune sialoadenitis is characterized by the presence of focal lymphocytic infiltrates in the SMG, which is most likely preceded by activation of autoreactive lymphocytes. Dendritic cells are unique among the antigen presenting cells (APC), in that they are the only cells, suited to activate naïve T lymphocytes (20, 21). Whereas immature dendritic cells have a capacity to phagocytose and process antigen, increased levels of costimulatory and MHC class II molecules are expressed upon maturation, enabling the cells to optimally present antigen to T cells (22).

In chapter 2, we demonstrated that the development of sialoadenitis in the NOD mouse is preceded by an influx of dendritic cells into the SMG. This influx was in part lymphocyte

independent, occurred between 2 and 5 weeks of age, and was not observed in C57BL/10 or BALB/c mice. Similar observations have been done in the pancreas of the NOD mouse, in which the development of insulitis was preceded by the accumulation of dendritic cells (23, 24). This accumulation correlated well with the expression of TNF-α, which has been shown to be involved in the pathogenesis of diabetes in the NOD mouse (24). Also in other experimental autoimmune models, an influx of dendritic cells into the target organ of the autoimmune reaction has been demonstrated to precede the development of lymphocytic infiltrates (25-28). In the BioBreeding (BB) rat model of autoimmune thyroiditis, this influx was followed by cluster formation between dendritic cells, T lymphocytes, and B lymphocytes (25, 28). In an experimental autoimmune encephalitis (EAE) model, a close correlation was observed between the immigration of dendritic cells into the central nervous system, the mRNA expression of a dendritic cell specific MHC class II transactivator (CIITA form I), expression of MHC class II molecules, and the onset of disease (26). This correlation could indicate a crucial role of dendritic cells in the initiation of EAE. The accumulation of dendritic cells in this model was preceded by a macrophage influx. As the influx of macrophages was not accompanied by increased expression of MHC class II or of CIITA form IV, which is indicative for APC activity of macrophages, it was suggested that these macrophages might serve to attract dendritic cells by the secretion of chemokines (26).

The capability of dendritic cells to indeed start off an autoimmune reaction was demonstrated in several experiments. It was found that dendritic cells, expressing an immunodominant epitope of the lymphocytic choriomeningitis virus glycoprotein (LCMV-GP), could induce diabetes in transgenic mice expressing the LCVM-GP under control of the rat insulin promotor (29). Furthermore, splenic dendritic cells, pulsed with thyroglobulin *in vitro*, or isolated from mice in which thyroiditis was induced, could induce thyroiditis in recipient mice (30). Also in the development of EAE, dendritic cells, expressing an epitope of myelin basic protein, were capable of initiating the autoimmune reaction (31).

In addition to a role of dendritic cells as APC for autoreactive lymphocytes, they may also contribute to the development of an autoimmune response by the production of proinflammatory cytokines, thereby creating an environment in which an autoimmune reaction is prone to develop. Recently, NOD dendritic cells were demonstrated to exhibit increased activity of the transcription factor NF-κB (32). As NF-κB is an important regulator of proinflammatory cytokine expression (reviewed in 33), it can be envisaged that NOD dendritic cells express increased levels of these cytokines, which may result in enhanced recruitment and activation of leukocytes.

The early accumulation of dendritic cells into NOD-*scid* SMG demonstrated that the dendritic cell influx could occur in the absence of lymphocytes. However, the number of dendritic cells present in SMG of 5-week-old NOD-*scid* mice was smaller than in age-matched NOD mice. Similar observations were done in the pancreas of the NOD mouse when compared to NOD-*scid* (23). The explanation for these observations may reside in the reduced function of NOD-*scid* APC, due to the absence of lymphocytes in this mouse strain. The function of the dendritic cells as well as the phenotype of these cells is regulated by cross-

talk with lymphocytes. Although the isolation of similar numbers of APC from NOD and NOD-*scid* islets has been described, the capacity of NOD-*scid* islet APC to stimulate T cell clones was weak when compared with the NOD islet APC (34). This indicates that dendritic cells indeed depend on T cell derived signals in order to acquire the phenotype optimal for antigen presentation.

In conclusion, the detection of significant numbers of dendritic cells in the NOD SMG prior to the development of sialoadenitis may indicate an important role of these APC in the initiation of sialoadenitis. In addition to their role in antigen uptake and presentation to autoreactive T lymphocytes, aberrant function of NOD dendritic cells may as well play a role. However, the mere presence of dendritic cells in the NOD SMG only provides circumstantial evidence that the primary presentation of autoantigen is excerted by these dendritic cells. Additional experiments are needed in which the exact role of these cells in the early phase of sialoadenitis is addressed.

These experiments could include the isolation of dendritic cells from the preautoimmune NOD SMG, followed by the isolation and identification of the predominant peptide(s), present in the MHC class II molecules. This approach has been followed to identify self peptides, expressed on MHC class II molecules of MRL/lpr lymph node cells, and of NOD splenic leukocytes (35, 36). After identification, this peptide could be generated in large quantities, to be used for the immunization of young NOD mice in an attempt to influence the development of sialoadenitis. In addition, SMG derived dendritic cells could be used in co-culture experiments with T cell clones, generated from SMG of old NOD mice. These experiments would not only elucidate the role of dendritic cells as APC in the initiation of sialoadenitis, but could also help in the identification of the primary antigen(s).

Furthermore, it would be of interest to examine whether prevention of the influx of dendritic cells into the NOD SMG could delay or prevent the development of sialoadenitis. Actually, this may be hard to accomplish, since systemic administration of an antibody aimed at preventing dendritic cell influx, most likely also influences dendritic cells at other locations, such as the peripheral lymphoid organs, that will affect the outcome of the experiment.

Aberrances in NOD neonatal SMG: basis for the development of sialoadenitis?

The accumulation of dendritic cells may be due to an intrinsic, genetically programmed abnormality in the NOD and NOD-*scid* SMG that results in disturbed glandular homeostasis. Altered expression of a variety of proteins has been described in the SMG of NOD and NOD-*scid* mice, before as well as following the onset of sialoadenitis, which may contribute to the attraction of dendritic cells towards the SMG (37-40). However, most alterations were observed in aged mice, suggesting that, although they also occurred in NOD-*scid* mice - in the absence of adaptive immune autoaggression - a direct contribution of these aberrances to the attraction of dendritic cells before 5 weeks of age is unlikely. Still, it can be envisaged that altered protein expression in SMG of aged mice is the outcome of an underlying glandular abnormality, which may have developed earlier in life, perhaps even during embryogenesis, and is directly responsible for the influx of dendritic cells.

For this reason, the abnormalities in neonatal NOD and NOD-derived SMG, described in chapter 5, are of special interest. Reduced acinar cell populations, increased acinar cell volumes, and reduced proliferation were observed, which was accompanied by an altered morphology of the NOD neonatal SMG. These abnormalities were normalized by 8 days after birth. Increased expression of the apoptosis related molecules Fas, FasL and bcl-2 was observed at three days of age, which continued to be elevated in SMG of NOD and NOD-*scid* mice throughout life, as was described in chapter 4. Furthermore, whereas type IV collagenase activity (matrix metalloproteinase (MMP)-2 and MMP-9) was reduced in SMG of NOD and NOD derived mice at one day of age, significantly increased activity was observed at three weeks of age when compared with control mice (chapter 5).

These observations indicate that abnormalities occur in NOD SMG from 1 day of age onwards. Although some of these aberrances are normalized before the age of three weeks, others are not. Abnormalities have also been demonstrated in the pancreas of neonatal NOD and - to a lesser extent - NOD-*scid* mice. Pancreata of 1-day-old NOD mice have an increased percentage of small islets of Langerhans, whereas the glucagon positive area in these islets is increased when compared with C57BL/6 mice. It was suggested that these data could indicate that at birth, NOD pancreata contain an increased percentage of immature islets of Langerhans (41).

As dendritic cells have been demonstrated to be capable of acting upon metabolic properties of endocrine cells (42, 43), it can be envisaged that their influx serves to influence the metabolism of glandular components in an attempt to restore glandular homeostasis. In the pancreas of the NOD mouse, hyperactive islets of Langerhans have been described, before the development of lymphocytic infiltrates, that were primarily associated with the accumulation of dendritic cells (23). Furthermore, isolated NOD islets contained increased insulin levels as compared with control islets. Experiments in which NOD islets were cocultured with NOD splenic dendritic cells resulted in the induction of insulin release from the islets (thesis J.G.M. Rosmalen, Erasmus University Rotterdam). These experiments demonstrate that NOD dendritic cells can influence the insulin producing ß-cells. Although the influx of dendritic cells may be aimed at normalizing glandular aberrances, defects in NOD dendritic cells could perhaps result in exacerbation of existing abnormalities.

In addition to an influence on metabolic properties of epithelial cells, dendritic cells and macrophages have also been postulated to influence interactions between mesenchymal and epithelial cells during glandular development (44). Macrophages have actually been shown to be involved in mouse mammary gland development by regulating branching morphogenesis (45). Altered activity of MMP-2 and MMP-9 during the first three weeks of life can result in disturbed interactions between SMG epithelial cells and extracellular matrix components, which dendritic cells, upon arrival in the NOD SMG, may also attempt to restore. In conclusion, the appearance of dendritic cells in the NOD SMG before the initiation of sialoadenitis may result from an intrinsic glandular abnormality, existing already at birth, and perhaps even developing during embryogenesis.

Although the primary role of the accumulation of dendritic cells in the NOD SMG per-

haps is to restore the glandular abnormalities, defects in NOD dendritic cells may account for the opposite. Therefore, additional experiments delineating the cross-talk between the glandular components and the dendritic cells are indicated.

First, the presence of dendritic cells in SMG of NOD.B6*Idd3*, NOD.B10*Idd5*, and NOD.B6*Idd3*.B10*Idd5* congenic mice could be examined. In these strains, the diabetes susceptibility genes *Idd3* and *Idd5* are replaced by the corresponding intervals derived from C57BL/6 and C57BL/10 mice, respectively, which was shown to influence the development of sialoadenitis (46). Whereas trends of normalization towards the C57BL/6 phenotype were observed in the sialoadenitis of NOD.B6*Idd3* and NOD.B10*Idd5* congenic mice, double congenic mice exhibited a significantly reduced pathophysiology as compared with the NOD mouse. Aberrances in neonatal SMG were also reduced in the NOD.B6*Idd3*.B10*Idd5* congenic mice. It would therefore be of great interest to examine whether restoration of the developmental pattern and the influence on the development of sialoadenitis are inversely associated with decreased numbers and/or functional activity of dendritic cells, accumulating in the SMG. This would shed light on the influence of aberrances in SMG development on the influx of dendritic cells. In any case, the molecular event(s), directly responsible for the dendritic cell influx, still needs to be identified since aberrant development may influence the expression of many factors that may attract dendritic cells.

Second, SMG epithelial cell cultures of NOD mice could be used to study the intrinsic properties of the epithelial cells, such as proliferation and expression and secretion of mediators (including cytokines, chemokines, extracellular matrix components, and ECM degrading enzymes). This would enable comparison with epithelial cells of control SMG and identification of factors that are abnormally expressed *in vitro*. Subsequently, coculture experiments of epithelial cells with dendritic cells, isolated from the spleen or from the SMG of NOD mice should be performed to examine if dendritic cells could influence the properties of the epithelial cells. This could also reveal whether the dendritic cell influx can restore the glandular homeostasis.

Apoptosis is neither involved in the initiation, nor in the effector phase of sialoadenitis in the NOD mouse

Apoptosis has classically been regarded as a mechanism of physiological cell death, not inducing an immune response, in contrast to cell death by necrosis. However, evidence has accumulated suggesting that apoptosis may contribute to the initiation of (auto)immune responses. The induction of apoptosis could result in the exposure of nuclear or cytosolic antigens, normally not encountered by lymphocytes, or in the generation of cryptic antigens via the activation of enzymes, activated following the induction of apoptosis (47, 48). Furthermore, apoptosis has been described to induce maturation of dendritic cells, and the release of pro-inflammatory cytokines by these cells (49). This may be mediated by the release of genomic DNA, which has been shown to induce the maturation of macrophages and dendritic cells (50). Under normal conditions, apoptotic cells are cleared with great efficiency, and it has been suggested that an autoimmune response may only develop if defects

in clearance occur, due to defective phagocytosis or increased apoptosis (51).

In chapter 4, we describe the presence of apoptotic cells in SMG of NOD, NOD-*scid* and control mice before the development of lymphocytic infiltrates. Apoptotic cells were detected in all SMG, and their numbers in SMG of 5-week-old NOD and NOD-*scid* mice did not exceed those in control mice. In all mouse strains, we noticed an increased number of apoptotic cells in glands of 3-day-old mice as compared with the number at 5 weeks of age, which is likely to reflect extensive remodelling of the neonatal SMG, as this involves both proliferation and apoptosis. Although these results do not support a role for apoptosis in the initiation of sialoadenitis in the NOD mouse, we did detect increased expression of Fas, FasL, and bcl-2 in NOD and NOD-*scid* SMG as early as 3 days of age. The expression of bcl-2 may explain the absence of increased numbers of apoptotic cells, despite increased expression of Fas and FasL.

In a human salivary gland epithelial cell line stably transfected with bcl-2 or bcl-X_L, Fas mediated apoptosis was significantly inhibited (52). Despite the inhibition of apoptosis, activation of caspase-3 was observed, and the cells were unable to respond normally to the muscarinic acetylcholine receptor agonist carbachol. This indicates that, although the induction of apoptosis was prevented by anti-apoptotic molecules, the cells remained functionally abnormal (52). If this also occurs in the SMG of the NOD mouse, this could contribute to the decreased stimulated saliva production. However, a decreased secretory response has not been observed in NOD-*scid* mice, suggesting that if the expression of Fas and FasL in the NOD and NOD-*scid* SMG would result in functional impairment of the epithelial cells, this does not account for a major decrease in salivary secretion. It remains possible that activation of caspase-3, although not resulting in apoptosis of the cell, may cleave particular cellular substrates, leading to the generation of cryptic antigens. Therefore, it would be of interest to examine levels of active caspase-3 in NOD and NOD-*scid* SMG.

Disturbed glandular expression of Fas, FasL, and bcl-2 in the NOD SMG could result from altered glandular homeostasis, the basis of which may already be present at birth. It may be a primary defect, or occur secondary to another abnormality, such as increased expression of matrix metalloproteinases. Although the function of Fas, FasL, and bcl-2 is usually considered within the context of apoptosis, it has previously been suggested that molecules, involved in the regulation of apoptosis may exert functions beyond that of cell death. For example, bcl-2 has been shown to reduce the proliferation of thymocytes and delay the cell cycle entry of mitogen-stimulated B and T lymphocytes, and of resting fibroblasts, a function that could be separated from its anti-apoptotic effect (53, 54). Ligation of Fas on dendritic cells, which are resistant to the induction of Fas-mediated apoptosis, was shown to induce their maturation and release of proinflammatory cytokines (55). Similarly, Fas on dendritic cells or on epithelial cells in the NOD SMG, could induce activation of these cells and the release of IL-1ß and TNF-α upon ligation. Also in T lymphocytes, Fas-FasL interaction can induce activation and proliferation, depending on the state of activation of the T cells (56). Although speculative, this might occur in the NOD SMG once lymphocytic infiltrates have developed. In summary, abnormal expression of apoptosis related molecules in NOD and

NOD-*scid* SMG could be the outcome of altered glandular homeostasis, perhaps contributing to the early phase of the autoimmune response through the activation of caspase-3, or through the activation of SMG epithelial or dendritic cells.

When lymphocytic infiltrates had developed in the NOD SMG, numbers of apoptotic cells were not increased in these SMG as compared with control mice, despite the presence of activated T lymphocytes expressing FasL. A similar situation is found in thyrocytes of patients with Hashimoto's thyroiditis (HT) and of patients with Graves' disease (GD), which were both shown to express Fas and FasL (57). Although HT thyrocytes were often apoptotic, as demonstrated in thyroid sections, GD thyrocytes were not. Furthermore, isolated thyrocytes of GD patients were resistant to the induction of apoptosis. It was shown that the anti-apoptotic molecules bcl-X_l and cFLIP (Fas-associated death domain-like IL-1ß-converting enzyme-inhibitory protein) were upregulated in GD thyrocytes (57). Cytokines were demonstrated to affect the expression of apoptosis regulatory proteins, indicating that the presence of an inflammatory infiltrate may influence the susceptibility of cells to the induction of apoptosis.

In patients with Sjögren's syndrome, Fas-induced apoptosis has been regarded as an important mechanism, responsible for the induction of damage to salivary gland epithelial cells (3-5, 58). In addition, apoptosis was postulated to be responsible for the generation of 120 kD α-fodrin, an autoantigen in Sjögren's syndrome (59). Also in the NOD mouse, evidence has been presented suggesting that apoptosis is an important mechanism in the effector phase of sialoadenitis (60, 61). However, controversy remains on this issue, both in patients with Sjögren's syndrome, and in the NOD mouse.

Chemokine expression in NOD SMG: limited role in initiation, but contribution to exacerbation of sialoadenitis?

The traffic of dendritic cells is regulated by the coordinated expression of chemokine receptors on their cell surface. While immature dendritic cells express receptors for inducible chemokines, especially expressed at sites of inflammation, mature dendritic cells express receptors for constitutive chemokines, expressed in peripheral lymphoid organs (62-64). In chapter 6 we studied whether the accumulation of dendritic cells in the NOD and NOD-*scid* SMG could be the result of alterations in the expression of chemokines. No difference was found in expression of MIP-1α, MCP-1, and RANTES in SMG of NOD, NOD-*scid*, and control mice before the development of lymphocytic infiltrates, thus not supporting a role for differential expression of these chemokines in the attraction of dendritic cells. However, the expression of other chemokines that can attract immature dendritic cells, such as MIP-1ß and MCP-3 (64), remains to be investigated. MIP-1ß mRNA expression in SMG of the various mouse strains was not revealed by RNAse protection assay, suggesting that this chemokine also does not contribute to the differential dendritic cell influx.

The expression levels of MCP-1 and MIP-1α may not be high enough to result in the attraction of dendritic cells, since no dendritic cells were identified in the SMG of control mice. The possibility remains that altered expression of chemokine receptors on NOD mono-

cytes/ dendritic cells results in an increased responsiveness of the dendritic cells to the expressed chemokines and the accumulation of DC in the SMG. After exposure of NOD dendritic cells to IL-12, anti-CD40, or TNF-α, increased activation of the transcription factor NF-κB was observed when compared with control mice (32). Hyperactivation of this transcription factor may, directly or indirectly, influence the expression of chemokine receptors. Therefore it would be of great interest to compare the expression of chemokine receptors on dendritic cells of NOD, NOD-*scid* and control mice.

Messenger RNA expression of MIP-1α, MCP-1, RANTES and IP-10 was altered following the development of lymphocytic infiltrates in the submandibular glands of the NOD mouse (chapter 6). Increased mRNA expression of MIP-1α and MCP-1 was accompanied by increased protein levels, measured in SMG lysates. As an increased expression of these chemokines was not observed in the SMG of NOD-*scid* mice, their expression is probably due to the lymphocytic infiltrates. The expression pattern of these chemokines likely reflects the predominant presence of Th1 cells within the infiltrates. The Th1 cells may themselves be responsible for the alterations in chemokine expression. T lymphocytes in minor salivary glands of patients with Sjögren's syndrome have been shown to produce MIP-1ß, MIP-1α, and RANTES (65). Lymphocytes infiltrating the lacrimal gland of the NOD mouse were shown to express IP-10 and RANTES mRNA (66). Furthermore, Th1 cells isolated from the pancreas of the NOD mouse expressed MIP-1α, MCP-1, and low levels of IP-10 and RANTES upon restimulation with anti-CD3 *in vitro* (67).

Next to chemokines, the infiltrating lymphocytes release cytokines such as TNF-α, IL-1ß, and IFN-γ, which may influence the expression of chemokines by cells surrounding the lymphocytic infiltrates. Dendritic cells can also be responsible for the altered chemokine expression as different stimuli have been shown to induce expression of MIP-1α, RANTES, and MCP-1 by these cells (68, 69). Detailed immunohistochemical studies need to be performed to identify the various cell type(s), responsible for the production of the chemokines.

Regardless of the origin, the chemokines expressed in SMG of NOD mice following the onset of lymphocytic infiltration can attract additional leukocytes. Leukocytes that express receptors for MIP-1α, RANTES, MCP-1, and IP-10 include Th1 cells, macrophages and dendritic cells (70). This indicates that, once the lymphocytic infiltrates have started to develop, they may directly or indirectly contribute to the attraction of additional leukocytes and influence the course of the autoimmune response. In the lacrimal gland of the NOD mouse, the coordinate expression of RANTES, IP-10, and the receptors for these chemokines on T cells, CCR1, CCR5, and CXCR3, provided evidence that these chemokines indeed contributed to the selective recruitment of lymphocytes into the gland (66). The role of individual chemokines in the late phase of sialoadenitis can be examined by treatment of mice with blocking antibodies to these chemokines. However, if the effect of such an experiment would be limited, this could be due to redundancy of the chemokine system. In that case, additional experiments should be performed using combinations of antibodies to various chemokines and/or chemokine receptors.

Two types of sialoadenitis in two mouse models for Sjögren's syndrome

When the development of sialoadenitis in the NOD and MRL/lpr mouse models was studied in time, remarkable differences were observed (chapter 2, and Table 1). First, sialoadenitis in MRL/lpr mice developed at an earlier age as compared with the NOD mouse. Second, the lymphocytic infiltrates developed in the MRL/lpr SMG in the absence of a preceding influx of dendritic cells. Third, whereas the lymphocytic infiltrates in the NOD SMG gradually organized, infiltrates in the SMG of MRL/lpr mice were organized at time of first appearance, and this organization was lost over time. Loss of organization of the infiltrates in the MRL/lpr SMG was accompanied by invasion of the glandular parenchyma. Fourth, the infiltrates in the MRL/lpr SMG contained high numbers of $BM8^+$ macrophages, while these cells were less frequent in NOD SMG.

Table 1. Histopathological features of sialoadenitis in NOD and MRL/lpr mice

	NOD mouse	MRL/lpr mouse
First appearance of infiltrates	10 weeks	5 weeks
Influx of dendritic cells prior to development of focal infiltrates	Yes, between 2-5 weeks of age	No
Organized lymphocytic infiltrates		
- at first appearance	No	Yes
- at 20 weeks	Yes	No
Appearance of lymphocytic infiltrates at 20 weeks	Focal, well defined	Invading the surrounding glandular parenchyma
Presence of $BM8^+$ macrophages	Thin rim around the infiltrate	Thick rim around the infiltrates, and within the infiltrates

These two patterns of sialoadenitis development remarkably resemble the differences reported between the development of insulitis in the spontaneous NOD model and the BDC2.5 TCR transfer model (71). In the latter model, transfer of the diabetogenic $CD4^+$ T cell clone BDC2.5 to young (< 3 weeks) NOD mice resulted in the rapid and simultaneous accumulation of APC and lymphocytes, whereas in the untreated NOD mouse, the infiltration of lymphocytes was preceded by a dendritic cell influx. In the transfer model, the development of insulitis and diabetes occurred with accelerated kinetics and was accompanied by extensive infiltration of $BM8^+$ macrophages. Furthermore, the infiltrates that developed did not show any structural organization. It was suggested that in the NOD BDC2.5 TCR transfer model, an increased frequency of autoreactive lymphocytes is present as compared with the spontaneous NOD model. These lymphocytes would perhaps not require activation in the pancreas draining lymph node by APC loaded with pancreas derived antigen, which could explain the development of insulitis in the absence of a preceding influx of dendritic cells (71).

The presence of a significant number of T lymphocytes in the SMG of 1-week-old

MRL/lpr mice may indicate a similar scenario in the SMG of the MRL/lpr mouse. It can be envisaged that, due to the *lpr* mutation, a high percentage of autoreactive lymphocytes is present in the periphery, which are directed to an autoantigen also present in the SMG, but not primarily SMG derived. Activation of these autoreactive T cells would not be restricted to the SMG draining lymph node, and could therefore occur in the absence of antigen presentation by SMG derived APC. This would then result in the development of sialoadenitis which is not preceded by the accumulation of dendritic cells.

The presence of $BM8^+$ macrophages during the development of insulitis correlated with a rapid destruction of ß-cells (71). The infiltrates, present in the SMG of MRL/lpr mice invaded the surrounding glandular tissue, and were not demarcated, as observed in the NOD SMG. Furthermore, epithelial cells in the vicinity of the lymphocytic infiltrates appeared damaged, suggesting that a destructive type of sialoadenitis occurred in the MRL/lpr SMG.

The presence of $BM8^+$ macrophages could serve several purposes. First, it is possible that these cells induce damage to the SMG epithelial cells. Second, their presence might serve to remove cellular debris of damaged glandular epithelial cells. Third, following the initiation of sialoadenitis, they could, perhaps in the process of removal of damaged cells, take up antigen and present this to autoreactive lymphocytes in the draining lymph nodes. This could lead to antigen spreading and aggravation of the autoimmune response. However, as high numbers of autoreactive lymphocytes may be present in the MRL/lpr mouse, it can be imagined that, similar to the initiation of sialoadenitis, aggravation of the autoimmune response occurs independently of additional antigen presentation in the SMG draining lymph node.

The difference observed in the degree of organization of the lymphocytic infiltrates in the SMG of both mouse models could be due to the source of the autoantigen(s) in both mouse strains. In the NOD mouse, in which the development of sialoadenitis is accompanied by characteristics of organ-specific autoimmune disease as opposed to the MRL/lpr mouse, showing characteristics of systemic autoimmunity, continued release of antigen from the salivary glands may result in the development of structured infiltrates. Repetitive injections of dendritic cells, loaded with an immunodominant epitope of an antigen transgenically expressed in the pancreas of a mouse model for diabetes, resulted in the development of organized lymphoid structures in the pancreas (29). This suggests that, if SMG derived antigen is continuously released, this could result in the formation of organized infiltrates.

In the MRL/lpr mouse on the other hand, the source of the primary autoantigen may be elsewhere in the body than in the salivary gland. Aggravation of the autoimmune response may not be the result of the release of antigens from the salivary gland. Instead, it may be due to uncontrolled activation of autoreactive lymphocytes in the course of the systemic autoimmune response occurring in this mouse strain. Although in this scenario activation of autoreactive lymphocytes is postulated to occur mainly in secondary lymphoid tissues, evidence has recently been presented suggesting that regulation of autoreactive cells in MRL/lpr mice also takes place in the target tissues themselves. It was found that MRL/lpr mice, deficient in ß2-microglobulin, suffered from accelerated skin disease whereas nephritis was ameliorated (72). Regulation of the autoimmune response at the level of the target organ, perhaps also

occurring in the SMG, may explain this divergent effect in skin and kidney.

Patients with Sjögren's syndrome can suffer from a variety of disease manifestations, depending on the involvement of other organs in addition to the salivary and lacrimal glands. Moreover, it may occur in the presence or absence of an additional autoimmune disease, such as rheumatoid arthritis or systemic lupus erythematosus. The list of factors implicated in the pathogenesis of Sjögren's syndrome is long, suggesting that different pathogenetic processes play a role in the initiation and effector phase of the disease. The involvement of pathogenetic factors may vary from patient to patient, as do the disease manifestations.

The development of sialoadenitis in the NOD and the MRL/lpr mouse models probably represent two different pathogenetic mechanisms. The type of sialoadenitis developing in the NOD mouse may predominantly reflect an organ-specific autoimmune process, in which the primary antigen resides in the exocrine glands. Similarly, the development of sialoadenitis in patients with the NOD-type sialoadenitis is structured, and not accompanied by destruction of glandular tissue. Instead, anti-M3 muscarinic acetylcholine receptor antibodies may be present in these patients, mediating a decreased secretory output.

The sialoadenitis in another group of patients has similarities with the MRL/lpr mouse model. These patients suffer from other autoimmune manifestations, and the primary antigen may be general, such as antigens released by cells dying from apoptosis. The development of sialoadenitis is unstructured, and accompanied by destruction of glandular tissue induced by apoptosis or through the release of cytotoxic mediators from inflammatory cells. Despite parenchymal destruction, a decreased secretory response is not observed in the majority of these patients, until glandular destruction has severely progressed.

Interestingly, the existence of two pathogenetic mechanisms in Sjögren's syndrome has recently been proposed by others. Stimuli from the epithelial cells were placed central in the activation and retention of lymphocytes in one mechanism, whereas in the second mechanism, dysregulation of systemic T cell homeostasis was proposed to enhance the ability of activated circulating lymphocytes to migrate into the salivary glands (73).

It would be of interest to compare the development of sialoadenitis in more mouse models for Sjögren's syndrome, as each model may well represent only a subgroup of patients, and the existence of other types of sialoadenitis can be envisaged. This might offer great insight into the different types of sialoadenitis and the underlying pathogenetic mechanisms. Furthermore, it would increase insight into mechanisms responsible for clinical disease manifestations in different patient groups, which could ultimately result in the development of subgroup specific therapeutic strategies.

Macrophages and dendritic cells are normal components of human minor salivary glands

Salivary gland epithelial cells have been suggested to actively participate in the initiation and perpetuation of the autoimmune reaction. The cells were found to express HLA-DR molecules and proinflammatory cytokines (74-78). Furthermore, the expression of the cos-

timulatory molecules CD80 and CD86 on the epithelium has been described (79, 80). It was proposed that interaction of costimulatory molecules on the epithelial cell with CD28 on the T cell, occurring simultaneously with antigen presentation in HLA class II molecules on the epithelial cells, could result in activation of the autoreactive T cells. In this scenario, the epithelial cells would function as non-professional APC.

We have demonstrated the presence of professional APC (RFD7$^+$ macrophages and L25$^+$ dendritic cells) not only in minor salivary glands in which a lymphocytic infiltrate was present, but also in noninfiltrated glands (chapter 3). RFD9$^+$ macrophages and CD1a$^+$ dendritic cells were specifically detected in minor salivary glands infiltrated by lymphocytes. Dendritic cells are capable of activating naïve T lymphocytes (20, 21), in contrast to non-professional APC. Therefore, a role for dendritic cells, but not for epithelial cells, in the initiation of sialoadenitis can be envisaged. Moreover, as epithelial cells can only contact T lymphocytes that may coincidentally infiltrate non-diseased glands, the chance of interaction with an autoreactive T lymphocyte is probably too low for such an event to occur.

The RFD7$^+$ macrophages may well represent a resident macrophage population, serving to remove debris from the glands. In a later phase of the autoimmune process these cells may also play a role in the reactivation of autoreactive T lymphocytes that have already encountered antigen. Once lymphocytic infiltrates have developed and inflammatory mediators are produced in the glandular environment, the expression of HLA class II and costimulatory molecules can be induced on glandular epithelial cells. Subsequently, the epithelium (in addition to the macrophages and dendritic cells) may contribute to deterioration of the autoimmune reaction by presentation of antigen to already activated T lymphocytes that have infiltrated the gland, sustaining their further T cell activation and proliferation *in situ*. Salivary gland epithelial cells, expressing CD86, were found capable of providing costimulation to CD4$^+$ T cells, leading to proliferation of the latter cell type. CD86 was shown to preferentially interact with CD28 and not with CTLA-4, and an important regulatory role of the epithelial cells in local immune responses in the salivary gland was proposed (81). However, the observation that CD80 and CD86 were only expressed on glandular epithelium of patients with severe sialoadenitis suggests that epithelial cell mediated antigen presentation may only occur in the progressive stage of sialoadenitis (80).

The presence of RFD9$^+$ macrophages and CD1a$^+$ dendritic cells in minor salivary glands in which a lymphocytic infiltrate is present may be of help in the histopathological diagnosis of sialoadenitis. Inadequate scoring of the minor salivary gland biopsy is a problem encountered in our study and also described by others (82). As dendritic cells and macrophages were detected, diffusely distributed in minor salivary glands in which a lymphocytic infiltrate was present, their detection may be easier for the pathologist than the detection of focal infiltrates, which can be easily missed in a minor salivary gland section. Therefore, we propose that a well-controlled investigation on the presence of RFD9$^+$ macrophages and CD1a$^+$ dendritic cells in a large cohort of patients may be worthwhile to assess the potential contribution of these cells to the histopathological diagnosis of Sjögren's syndrome.

Development of sialoadenitis in the NOD mouse: from initiation through effector phase

Aberrances in the NOD SMG and the immune system may both contribute to the development of sialoadenitis (Fig. 1). Abnormalities early in life, possibly already occurring during organogenesis, may lead to an influx of dendritic cells into the NOD SMG, in an attempt to restore glandular homeostasis. In the pancreas of the NOD mouse, the close association between the accumulation of dendritic cells and macrophages and the presence of hyperactive islets of Langerhans is indicative for the potential of dendritic cells to influence the metabolism of these cells (23, 42, 43). A similar role for dendritic cells in the NOD SMG can be envisaged, since several abberances have been described in neonatal NOD SMG. While some of these disturbances were normalized at 1 week of age, others remained abnormal, such as the increased expression of MMP-2, MMP-9, and the apoptosis related molecules Fas, FasL and bcl-2.

Although expression levels of chemokines, capable of attracting immature dendritic cells (MIP-1α, MCP-1) in SMG of 1-day through 7-week-old NOD mice were similar when compared with age-matched control mice, aberrances may exist in the expression of chemokine receptors on NOD dendritic cells. Therefore, a role for chemokines in the accumulation of dendritic cells in the NOD SMG cannot be ruled out. Following the influx of dendritic cells into the SMG, they may be induced to mature by cytokines such as IL-1ß or TNF-α. These cytokines are known for their capacity to induce maturation of dendritic cells (83, 84). As a result, antigen acquired in the SMG can be retained, processed and presented to autoreactive T lymphocytes in the draining lymph nodes. In the lymph node, significant numbers of autoreactive lymphocytes may be present, which have accumulated there due to failure of central or peripheral tolerance mechanisms.

Abnormalities in the thymus of NOD mice may result in defective central tolerance. Changes in the thymic microenvironment have been demonstrated, such as increased perivascular spaces and alterations in the thymic epithelial network (85, 86). It has been demonstrated that a subset of immature thymocytes ($CD4^{-/lo}CD8^{+}$) in the NOD mouse displays decreased proliferation as compared with the C57BL/6 mouse. This was linked to the diabetes susceptibility locus *Idd6*, suggesting that it could also effect diabetes pathogenesis (87).

The contribution of thymic selection to the development of autoimmune responses in the NOD was demonstrated in experiments in which transplantation of NOD thymic epithelium to nude C57BL/6 mice resulted in the development of insulitis and sialoadenitis in the recipient mice (88). It should be emphasized that the recipient mice were 10-22 months of age at sacrifice, while the presence of inflammatory lesions has been described in multiple organs of 6-month-old C57BL/6 mice (89). Similar findings were observed in other mouse strains in which the MHC class II gene I-E was defective, when examined at 8 months of age (90). This indicates that, although the NOD thymic epithelium likely contributes to the development of autoimmune lesions in the C57BL/6 mouse strain, other C57BL/6 derived factors probably also add to the development of autoimmune responses in the recipient mice (88).

It has been reported that peptide-binding properties of NOD-specific $I\text{-}A^{g7}$ MHC

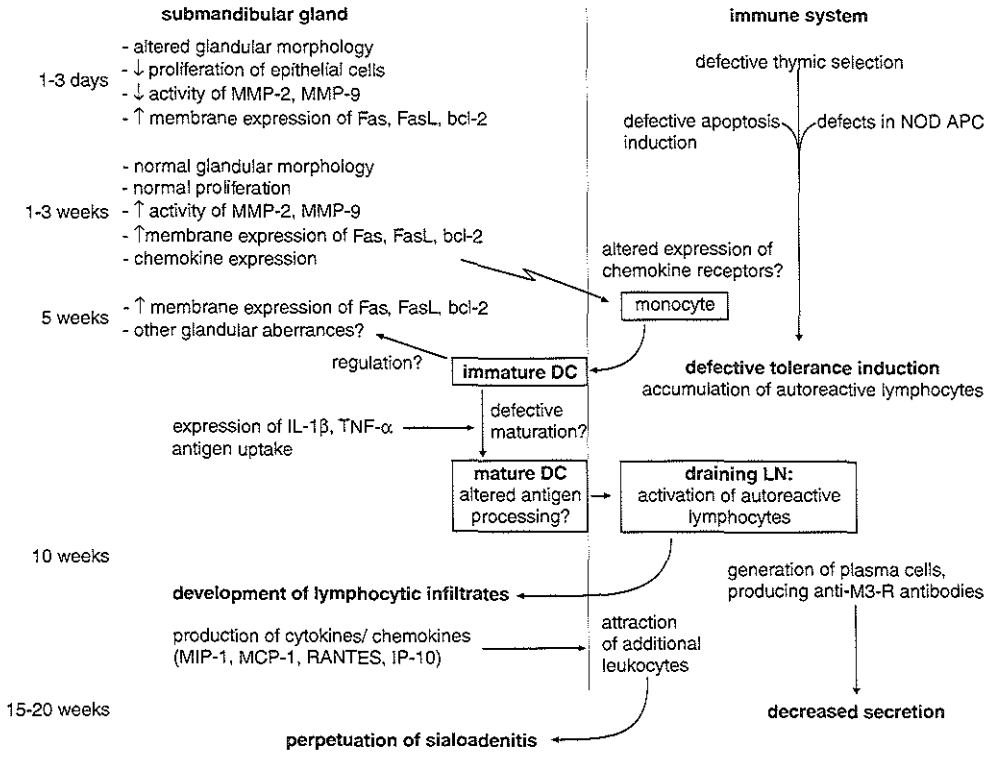

Figure 1
Aberrances in the NOD SMG and the immune system may both contribute to the development of sialoadenitis (explained in text).

class II molecules are poor, resulting in a short half-life of the MHC class II-peptide complexes (91). Furthermore, class II-associated invariant chain peptides (CLIP), which are complexed with MHC class II molecules until displaced by antigen-derived peptides, were present on an increased percentage of NOD B cells as compared with B cells from control mice. This was suggested to result from the low affinity of the MHC class II molecules for peptides (92). These observations were done in splenic APC and in B cells, but they could be a feature of thymic epithelial cells as well. As the low peptide binding capacity of NOD MHC class II molecules will not only affect binding of foreign peptides, but also of self-peptides, it can be imagined that both central and peripheral tolerance induction are affected by this defect.

Another abnormality, described for I-A^{g7} molecules, is the unusually high degree of flexibility of the peptide binding groove. This results in alternative conformations of the MHC class II molecule, which can interact with the T cell receptor with different affinities (93). This may both affect T cell education in the thymus and T cell stimulation in the periph-

ery. Although the aberrances in the I-A^{g7} molecule can contribute to the development of sialoadenitis, their expression is not essential, since in NOD.B10.H2b mice, which express MHC I-A molecules other than I-A^{g7}, sialoadenitis and dacryoadenitis develop with similar characteristics as in the NOD mouse (94).

Evidence has been presented suggesting that peripheral tolerance induction in NOD mice is defective. In a transgenic NOD model in which the hemagglutinin (HA) molecule of influenza virus is expressed by pancreatic ß-cells (NOD-InsHA mice), high avidity HA-specific $CD8^+$ T cells were present in the periphery, whereas they were absent in BALB-InsHA mice (95). In BALB-InsHA mice, it has been shown that tolerance induction occurred in the pancreas draining lymph nodes and was followed by elimination of the autoreactive lymphocytes (96, 97).

Failure of peripheral tolerance induction could be the result of defects in NOD APC, including the expression of I-A^{g7} MHC class II molecules. These defects may lead to the preferential activation of autoreactive T lymphocytes that differentiate into effector cells as opposed to the activation of regulatory T lymphocytes. Abnormal activation of transcription factors, as has been described in NOD dendritic cells (32), may promote the activation of effector T lymphocytes. Defective activation of suppressor T cells has also been described in NOD mice (98). NOD dendritic cells were demonstrated to express very low levels of MHC class II molecules, CD80, CD86, and CD40, which, except for CD40, were not upregulated upon maturation (99, 100). Low expression of CD86 has been described on NOD macrophages and dendritic cells, which was not dependent on the MHC haplotype of the mice (101). This was shown to affect the ratio between CTLA-4 and CD28, which is normally increased upon T cell stimulation. Since CTLA-4 delivers a negative signal to the T cell, in contrast to CD28, a reduced increase in the CTLA-4/CD28 ratio will result in prolonged immune reactivity. In NOD T cells, the ratio between CTLA-4 and CD28 was not increased upon stimulation, and reduced expression of CD86 accounted for the observed effect (101).

NOD macrophages were found to be defective in the induction of antigen-specific T cell proliferation (102). Upon stimulation with antigen or LPS, NOD macrophages expressed lower levels of glutathione (GSH) as compared to macrophages of the diabetes resistant NOR mice. GSH is involved in the reduction of disulfide bonds, an essential step in the processing of antigen in the lysosomes (103, 104). As antigen specific stimulation by NOR macrophages was normal, it was suggested that defective antigen presentation in NOD macrophages was related to the lower GSH levels expressed in these cells (102).

In addition to the defects in thymic selection and in NOD APC, which may result in the accumulation of autoreactive lymphocytes, defects in apoptosis have been described in the NOD mouse that may also affect central and peripheral tolerance induction (105-108).

Defective tolerance induction to SMG derived antigens may result in the initiation of an autoimmune reaction, following the presentation of normal SMG derived antigens to autoreactive lymphocytes. Alternatively, altered processing of SMG derived antigen may result in the presentation of cryptic determinants of antigen(s) to T lymphocytes that normal-

ly do not encounter the antigen that they recognize. Antigen processing by dendritic cells can be affected by exposure of these cells to inflammatory cytokines. This was demonstrated in an experiment in which treatment of mouse dendritic cells with IL-6 resulted in altered processing of the antigen hen egg lysozyme, and the presentation of cryptic determinants of the antigen that were not presented by control dendritic cells (109). Similar events may occur in the SMG.

Regardless of whether the initiation of the autoimmune response results from recognition of normal antigen by autoreactive lymphocytes, or from the presentation of cryptic epitopes or altered antigen, T cell activation in the SMG draining lymph nodes will most likely be followed by infiltration of the SMG. The newly arrived and activated lymphocytes may produce proinflammatory mediators such as cytokines and chemokines, resulting in the attraction of additional leukocytes. Dendritic cells may not only be essential in the processing and presentation of autoantigens to naïve T cells, but are perhaps also responsible for the attraction of activated T cells to the SMG. This may occur via the production of chemotactic factors such as MIP-1α, MIP-1ß, RANTES, and MCP-1, the expression of which has been described upon maturation (69), or result from exposure of dendritic cells to proinflammatory cytokines or chemokines.

During the inflammatory process, B cells are likely to be activated as well, which can result in the generation of autoantibody producing plasma cells. These autoantibodies will include anti-M3 muscarinic acetylcholine receptor antibodies, which are able to induce a decreased secretory response in NOD mice (110, 111). Additional evidence for a role of autoantibodies in diminished salivary secretion was recently provided by the observation that NOD.IL-4 gene knockout mice do not develop a decreased secretory response, despite extensive infiltration of the SMG. Anti-M3 muscarinic acetylcholine receptor antibodies were absent in this mouse strain, suggesting that IL-4 is essential in the development of salivary dysfunction via an effect on the antibody formation (112).

Although apoptosis related proteins continue to be aberrantly expressed in the NOD SMG throughout life, the absence of increased numbers of apoptotic cells in glands in which sialoadenitis has fully progressed, does not favor a role for apoptosis in the decreased production of saliva.

In conclusion, we postulate the development of sialoadenitis in NOD mice to occur in two phases. In the asymptomatic phase, aberrances, existing in the SMG of NOD mice, may result in the accumulation of dendritic cells that can activate autoreactive lymphocytes in the draining lymph nodes. Although the primary abnormality may reside in the SMG, possibly already existing at birth, defects in the immune system are essential for in the development of the autoimmune response. This gradually develops in the symptomatic phase, ultimately resulting in the production of autoantibodies that mediate the decreased secretory response.

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Abbreviations

ACTH : adrenocorticotropic hormone
ANA : anti-nuclear antibodies
AP-1 : activating protein-1
Apaf : apoptotic protease-activating factor
APC : antigen presenting cell
AQP : aquaporin
ATD : autoimmune thyroid disease
BB : biobreeding
BCA : B cell attracting chemokine
BrdU : bromodeoxyuridine
BSA : bovine serum albumine
CCR : CC chemokine receptor
CDR : complementarity determining region
CHB : congenital heart block
CLIP : class II-associated invariant chain peptides
CNS : central nervous system
CS : chronic sialoadenitis
CTLA-4 : cytotoxic T lymphocyte antigen-4
CXCR : CXC chemokine receptor
CIITA : MHC class II transactivator
4-DAMP : 1,1-dimethyl-4-diphenylacetoxypiperidinium
DC : dendritic cells
DNA : deoxyribonucleic acid
EAE : experimental autoimmune encephalomyelitis
EBV : Epstein-Barr virus
ECM : extracellular matrix
EGF : epidermal growth factor
ELC : Epstein-Barr virus-induced gene 1 ligand chemokine
ELISA : enzyme linked immunosorbent assay
FADD : Fas-associated protein with death domain
FAK : focal adhesion kinase
FCS : fetal calf serum
FLS : focal lymphocytic sialoadenitis
GD : Graves' disease
GM-CSF : granulocyte-macrophage colony stimulating factor
GSH : glutathione
GVHD : graft-versus-host disease
HA : hemagglutinin
HCMV : human cytomegalovirus
HCV : hepatitis C virus
HIV : human immunodeficiency virus
HLA : human leucocyte antigen
HSG : human salivary gland
HT : Hashimoto's thyroiditis
HTLV : human T-lymphotropic virus
ICAM : intercellular adhesion molecule
IDDM : insulin-dependent diabetes mellitus
IFN : interferon
IGFBP : insulin-like growth factor binding protein
IHC : immunohistochemistry
IL : interleukin
IP-10 : interferon inducible protein-10
ISH : in situ hybridization
KCS : keratoconjunctivitis sicca
LC : lymphocytic choriomeningitis
LFA : lymphocyte function associated antigen
L-NMMA : L-N^G-monomethylarginine
LPS : lipopolysaccharide
Ltn : lymphotactin
MCP : monocyte chemotactic protein
MHC : major histocompatibility complex
MIP : macrophage inflammatory protein
MMP : matrix metalloproteinase
MS : multiple sclerosis
MSG : minor salivary gland
NATD : non-autoimmune thyroid disease
NF-κB : nuclear factor-κB
NHL : non-Hodgkin lymphoma
Nik : NF-κB inducing kinase
NLE : neonatal lupus erythematosus
NO : nitric oxide
NOD : nonobese diabetic
NOR : nonobese diabetes resistant
NOS : nitric oxide synthase
NSAID : non-steroidal anti-inflammatory drugs
NZB : New Zealand black
NZW : New Zealand white
PARC : pulmonary activation regulated chemokine
PBC : primary biliary cirrhosis
PBS : phoshate buffered saline
PCD : programmed cell death
PKC : protein kinase C
PSP : parotid secretory protein
QNB : quinuclidinyl benzilate
RANTES : regulated upon activation, normal T cell expressed and secreted
RF : rheumatoid factor
RNA : ribonucleic acid
RNP : ribonucleoprotein
RPA : RNAse protection assay
RQ-PCR : real time quantitative polymerase chain reaction
RT-PCR : reverse transcriptase-polymerase chain reaction
scid : severe combined immunodeficiency
SEM : standard error of the mean
SjS : Sjögren's syndrome
SLC : secondary lymphoid organ chemokine
SLE : systemic lupus erythematosus
SMG : submandibular gland
TCA : T cell activation chemokine
TCR : T cell receptor
TGF : transforming growth factor
TIMP : tissue inhibitor of metalloproteinase
TNF : tumor necrosis factor
TUNEL : terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
VLA : very late antigen
VCAM : vascular cell adhesion molecule
XIAP : X chromosome-linked inhibitor of apoptosis protein
yRNA : cytoplasmic RNA

Summary

Sjögren's syndrome is an autoimmune disease, in which the salivary and lacrimal glands are affected. Lymphocytic infiltrates are detected in these glands, and patients may suffer from dry mouth and dry eyes, due to insufficient production of saliva and tears. Knowledge on mechanisms that may lead to decreased exocrine secretion has increased over the past few years. In contrast, little is known about events that initiate the autoimmune reaction leading to this decreased secretion. Although studies on the pathogenesis of autoimmune diseases have mainly focussed on the role of the immune system, evidence is accumulating suggesting that the target organ of the autoimmune disease may contribute to its initiation. The contribution of exocrine gland-derived and immune-derived components to the initiation of the autoimmune reaction in the exocrine glands can be studied in detail in mouse models for Sjögren's syndrome, in which the glands can be examined from birth until the autoimmune process has fully developed.

In the studies described in this thesis we examined whether salivary gland derived components could play a role in the pathogenesis of sialoadenitis. When the development of sialoadenitis was studied in two mouse models for Sjögren's syndrome, the nonobese diabetic (NOD) mouse and the MRL/lpr mouse, remarkable differences were found, suggesting two different pathogenetic types of sialoadenitis (chapter 2). The development of lymphocytic infiltrates in the submandibular gland (SMG) of the NOD mouse, but not in the MRL/lpr mouse, was preceded by an accumulation of dendritic cells in these glands, occurring between 2 and 5 weeks of age. This accumulation was not lymphocyte dependent, as it was also observed in the NOD-*scid* mouse. As dendritic cells are potent antigen presenting cells, capable of activating naïve T lymphocytes, a role for these cells in the initiation of sialoadenitis in the NOD mouse can be envisaged.

Although the composition of the lymphocytic infiltrates in minor salivary glands of patients with Sjögren's syndrome has been studied intensively by several groups, the presence of professional antigen presenting cells in these glands has received little attention yet. In minor salivary glands of Sjögren's patients, of controls, and of patients fulfilling only the oral or ocular hallmarks of Sjögren's syndrome, dendritic cells and macrophages were present (chapter 3). Furthermore, in minor salivary glands with a focal lymphocytic infiltrate, we noticed the presence of $CD1a^+$ dendritic cells and $RFD9^+$ macrophages, scattered throughout the gland. These subsets were absent in glands without lymphocytic infiltrates, and may therefore be of help in the histopathological diagnosis of Sjögren's syndrome.

Because evidence is accumulating suggesting that apoptosis may contribute to the initiation of autoimmune reactions, we also studied the presence of apoptotic cells in SMG of NOD mice (chapter 4). Despite elevated expression of Fas and FasL in SMG of NOD and NOD-*scid* mice as compared with control strains, the numbers of apoptotic cells in glands of the different mouse strains were similar. This could be due to the concomitant elevated expression of bcl-2. Also following the development of lymphocytic infiltrates, numbers of

apoptotic cells in NOD SMG were not increased as compared with control mice. These results suggest that the role of apoptosis in the development of sialoadenitis in the NOD mouse is limited.

Murine SMG are functionally immature at birth and undergo extensive morphodifferentiation in the first three weeks of life. It can be hypothesized that disturbances in this process lead to altered glandular homeostasis and thereby evoke autoimmunity later in life. Therefore, NOD and NOD-*scid* SMG were studied from birth onwards until the age of three weeks, to examine glandular morphology and the expression of factors that can influence this morphology, like extracellular matrix (ECM) components and enzymes capable of degrading the ECM (chapter 5). Histological analysis revealed more wide connective tissue septa, and more distinct septation of neonatal NOD SMG as compared with controls. Proliferation rates of epithelial cells in NOD SMG were decreased at birth. The morphological abnormalities and the decreased epithelial cell proliferation were no longer found at one week of age. Whereas no differences were found in the expression of ECM components, decreased activity of matrix metalloproteinase (MMP)-2 and MMP-9 was observed in NOD SMG at birth. At three weeks of age, increased activity of these enzymes was measured in the NOD SMG as compared with control mice. These aberrances suggest that organogenesis of the NOD SMG is deviant. This aberrant glandular development may promote a subsequent autoimmune reaction, leading to the sialoadenitis.

Because chemokines are important components of the immune system, tightly regulating the traffic of a.o. dendritic cells, we examined their expression in the SMG of the NOD and the NOD-*scid* mouse, and compared this with control mice. No differences were observed in mRNA and protein expression of the chemokines MIP-1α and MCP-1 between SMG of NOD and control mice from 1 day onwards until 7 weeks of age. Therefore, the expression of these chemokines is probably not directly responsible for the influx of dendritic cells into the NOD SMG, although we cannot exclude that the expression levels or the activity of the corresponding chemokine receptors on NOD and control monocytes and/or dendritic cells differ. Following the onset of sialoadenitis in the NOD SMG, increased expression of MIP-1α and MCP-1, and induced expression of IP-10 were observed. As this was not observed in NOD-*scid* mice, this altered chemokine expression is likely due to the development of lymphocytic infiltrates in the NOD SMG. It might well be that the increased or induced expression of particular chemokines will influence the composition of the infiltrates, and contribute to the perpetuation of the autoimmune process.

Our studies indicate that the cellular components of the exocrine glands may actively contribute to the pathogenesis of Sjögren's syndrome. In the NOD mouse, impaired glandular homeostasis may be responsible for the accumulation of dendritic cells that was observed in the SMG. Although the precise role of the dendritic cells in the pathogenesis of the sialoadenitis remains to be established, the present studies indicate that these cells play a central role in the initiation of the autoimmune process. Further studies should aim at the precise delineation of this role.

Samenvatting voor niet-ingewijden

Het afweersysteem is erop gericht het lichaam te beschermen tegen bacteriën, virussen en andere micro-organismen. Wanneer een micro-organisme het lichaam is binnengedrongen, kan dit door cellen van het afweersysteem worden opgenomen, waarna het wordt gedood en verteerd. Deze cellen worden antigeen presenterende cellen genoemd, omdat ze deeltjes, of antigenen, van het micro-organisme naar hun oppervlak brengen en ze vervolgens laten zien aan andere cellen van het afweersysteem, zoals T cellen. Voorbeelden van antigeen presenterende cellen zijn dendritische cellen en macrofagen. Wanneer T cellen de vreemde deeltjes herkennen, kan dit leiden tot hun activatie. Er wordt dan gesproken van een immuunreactie. De geactiveerde T cellen kunnen vervolgens andere cellen doden, waaronder de cellen die, ingeval van een virusinfectie, met dit virus zijn geïnfecteerd. Daarnaast kunnen ze B cellen stimuleren tot het produceren van antistoffen die ook een belangrijke rol spelen bij het opruimen van het binnengedrongen micro-organisme.

Naast antigenen die afkomstig zijn van vreemde indringers, presenteren antigeen presenterende cellen ook deeltjes die afkomstig zijn van lichaamseigen cellen. De B cellen en T cellen hebben bepaalde moleculen, receptoren, op hun oppervlak waarmee ze onderscheid kunnen maken tussen vreemde en eigen moleculen. Hiermee wordt voorkomen dat een immuunreactie ontstaat die gericht is tegen lichaamseigen moleculen. Echter, een enkele keer maakt het afweersysteem een vergissing en richt het zich wel tegen een structuur van het eigen lichaam. Er ontstaat dan een zogenaamde autoimmuunreactie. Wanneer deze autoimmuunreactie leidt tot een chronische ontstekingsreactie en tot het ontstaan van ziekteverschijnselen, wordt er gesproken van een autoimmuunziekte. Voorbeelden van autoimmuunziekten zijn type 1 diabetes (waarbij de insuline-producerende cellen in de alvleesklier worden aangevallen en vernietigd), multiple sclerose (waarbij het zenuwstelsel wordt aangetast), en reuma (waarbij onder andere de gewrichten worden beschadigd). Het syndroom van Sjögren is een autoimmuunziekte die, alhoewel minder bekend dan de zojuist genoemde, voorkomt bij 1% van de populatie, met name bij vrouwen ouder dan 50 jaar. De autoimmuunreactie richt zich vooral tegen de speeksel- en traanklieren, maar er kunnen ook andere organen bij het ziektebeeld betrokken zijn. De chronische ontsteking die ontstaat in de speeksel- en traanklieren gaat gepaard met een verminderde productie van speeksel en traanvocht, waardoor de patiënten last krijgen van een droge mond en droge ogen.

Theoretisch gezien verloopt de ontwikkeling van het syndroom van Sjögren in twee fasen, die geleidelijk in elkaar overgaan. In de eerste fase worden de eerste autoreactieve T cellen geactiveerd en wordt de autoimmuunreactie op gang gebracht. De patiënt heeft dit niet in de gaten, aangezien er in deze vroege fase nog geen klachten zijn. In de tweede fase verergert de autoimmuunreactie en wordt de ontstekingsreactie in de speeksel- en traanklieren chronisch van aard. De productie van speeksel en traanvocht neemt af, er ontstaan klachten, en de patiënt zal een arts bezoeken die, na het uitvoeren van een aantal testen, de diagnose kan stellen.

Alhoewel het inzicht in mechanismen die mogelijk een rol spelen bij het ontstaan van de verminderde produktie van speeksel en traanvocht de laatste jaren sterk is toegenomen, is nog niet bekend waardoor de autoimmuunreactie wordt veroorzaakt. Tot nu toe heeft het onderzoek naar de factoren, die mogelijk betrokken zijn bij het ontstaan van het syndroom van Sjögren, zich met name gericht op mogelijke fouten van het afweersysteem. T cellen en de B cellen met receptoren die geen goed onderscheid kunnen maken tussen dat wat lichaamsvreemd en dat wat lichaamseigen is, kunnen er de oorzaak van zijn dat er een autoimmuunreactie optreedt. Het is echter ook mogelijk dat veranderingen, die ontstaan in cellen van de speeksel- of traanklieren, ervoor zorgen dat deze cellen een vreemd uiterlijk krijgen, waardoor ze door het immuunsysteem als lichaamsvreemd worden gezien. De speeksel- en traanklieren zouden op deze manier zelf de oorzaak kunnen zijn van het ontstaan van de autoimmuunreactie.

De vroege fase van het syndroom van Sjögren is lastig te bestuderen in patiënten, aangezien de diagnose soms pas jaren na het ontstaan van de autoimmuunreactie wordt gesteld. Daarom wordt voor onderzoek naar deze fase van het syndroom van Sjögren vaak gebruik gemaakt van proefdieren. Er bestaan verschillende muizenstammen waarin zich spontaan een chronische ontstekingsreactie in de speeksel- en traanklieren ontwikkelt. Twee van deze stammen zijn de NOD muis en de MRL/lpr muis. Beide stammen vertonen een ontstekingsreactie in de speeksel- en traanklieren vanaf een leeftijd van ongeveer 10 weken.

Het onderzoek, beschreven in dit proefschrift, heeft zich gericht op de mogelijke rol van componenten die zich in de speekselklier bevinden tijdens het ontstaan van sialoadenitis. Zo wordt de ontstekingsreactie in de speekselklier genoemd. Wij hebben aangetoond dat in de speekselklier van de NOD muis dendritische cellen aanwezig zijn vóór het ontstaan van de sialoadenitis. In controle muizenstammen troffen we deze cellen niet aan. Dendritische cellen spelen een belangrijke rol bij het activeren van T cellen. Alhoewel wij dat niet hebben aangetoond, kan verondersteld worden dat de dendritische cellen in de speekselklier van de NOD muis antigenen opnemen, waarmee ze naar de dichtstbijzijnde lymfklier reizen. In de lymfklier zijn veel T cellen en B cellen aanwezig en de dendritische cellen kunnen hier op zoek gaan naar een T cel die het antigeen, dat ze in de speekselklier hebben opgenomen, herkent. Als zo'n autoreactieve T cel gevonden wordt, kunnen ze deze activeren, waarmee de autoimmuunreactie start.

In de speekselklier van de MRL/lpr muis zijn geen dendritische cellen gevonden vóór het ontstaan van sialoadenitis. Het is mogelijk dat het mechanisme dat leidt tot het ontstaan van de autoimmuunreactie in deze muis anders is dan in de NOD muis. Ook in de late fase van het autoimmuunproces hebben we verschillen aangetoond tussen de beide muizenstammen. Het lijkt er daarom op, dat in deze twee muismodellen voor het syndroom van Sjögren twee typen sialoadenitis onderscheiden kunnen worden, die verschillen in zowel de vroege als in de late fase van het ziekteproces.

Vervolgens hebben wij onderzocht of in kleine speekselklieren, afkomstig uit de lip van patiënten met het syndroom van Sjögren, en van controles, ook antigeen presenterende cellen aangetoond konden worden. In de speekselklieren van Sjögren patiënten en van con-

troles troffen we inderdaad dendritische cellen en macrofagen aan. Tussen de speekselklieren van patiënten vonden we daarbij verschillen die mogelijk gebruikt kunnen worden bij het stellen van de diagnose van het syndroom van Sjögren.

De rest van het onderzoek heeft zich geconcentreerd op het bestuderen van factoren in de speekselklier van de NOD muis die betrokken zouden kunnen zijn bij het aantrekken van de dendritische cellen. Wij hebben aangetoond dat de speekselklier van de NOD muis al bij de geboorte afwijkend is. De klieren zien er anders uit, en de cellen van de speekselklier ondergaan minder celdelingen dan die van controlemuizen. Daarnaast komen bepaalde moleculen in een andere hoeveelheid voor op de cellen van de speekselklier van de NOD muis in vergelijking met de controles. Bepaalde afwijkingen waren niet meer waarneembaar op een leeftijd van 1 week, andere bleven abnormaal gedurende de hele ontwikkeling van sialoadenitis.

Uit ons onderzoek hebben wij geconcludeerd dat bepaalde componenten in de speekselklier inderdaad een rol kunnen spelen bij het ontstaan van het syndroom van Sjögren. Afwijkingen in de speekselklier veroorzaken waarschijnlijk de aantrekking van dendritische cellen, die vervolgens autoreactieve T cellen kunnen activeren. Andere onderzoeksgroepen hebben afwijkingen aangetoond in het afweersysteem van zowel de NOD muis als van patiënten met het syndroom van Sjögren. Wij veronderstellen daarom dat de droge mond bij patiënten met het syndroom van Sjögren wordt veroorzaakt door een samenspel van afwijkingen in de speekselklier en afwijkingen in het afweersysteem.

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Saskia

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