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Late effects of chronic graft-vs.-host disease in minor salivary glands

M. R. Alborghetti[1], M. E. P. Corrêa[2], R. L. Adam[1], K. Metze[1], F. L. Coracin[2], C. A. de Souza[2], M. L. Cintra[1]

Departments of [1]Pathology, Medical School; [2]Hematology and Blood Transfusion Center-State University of Campinas (UNICAMP), Campinas, Brazil

BACKGROUND: The established pathologic criteria for minor salivary gland (MSG) involvement in chronic graft-vs.-host disease (cGVHD) could play a role in monitoring response to therapy.
METHODS: We evaluated MSG sequential biopsies during cGVHD therapy in 14 allogeneic bone marrow transplantation (BMT) patients. Nine patients that did not develop GVHD after BMT entered the control group. Biopsies were examined using hematoxylin-eosin, Periodic acid-Schiff (PAS) and leukocyte common antigen staining.
RESULTS: A significant loss of PAS+ acinar volume was observed at the diagnosis of cGVHD as much as at the end of treatment when compared with the control group. In the second evaluation, the inflammatory infiltrate was still greater than control group.
CONCLUSIONS: The results suggest that persistent xerostomia after cGVHD treatment is because of maintenance of lymphocytic infiltrate and consequent absence of MSG secretory unit recovery. This data may be useful to provide improved insight into the histopathology of this organ involvement.
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Keywords: bone marrow transplantation; grades; GVHD; minor salivary gland; pathology; xerostomia

Introduction

Chronic graft-vs.-host disease (cGVHD) is a major late complication of allogeneic bone marrow transplantation (BMT) and is the principal cause of morbidity and non-relapse mortality. Oral symptoms are present in approximately 80% of patients with its extensive form (1). There has been a considerable amount of work carried out on salivary gland (SG) involvement with GVHD. In fact, the work by Gratwhol et al. (2) represents the first publication detailing a specific oral complication of allogeneic transplantation and more specifically, oral GVHD. Reports of both human and animal models reveal a mean reduction of 55–90% in the salivary flow rate of GVHD patients (3, 4) as well as various immunological and sialochemical associated changes (5, 6). Schubert et al. (7) observed that the severity of oral abnormalities appeared related to the severity of systemic cGVHD. The altered glandular function is secondary to chronic sialoadenitis (7–9). Improved survival rates have led to a continuously increasing number of cGVHD patients suffering from induced salivary insult (10). The severity of SG involvement can be evaluated at the moment of the diagnosis and in sequential lip biopsies, aiming to assess the results of cGVHD treatment and sequels. Although the oral cavity can be readily examined, no rigorous histologic description of late post-treatment response of SG has yet been described. The purpose of this work is to describe the cGVHD-related sequential histological alterations in minor salivary glands (MSG) after cGVHD treatment as much as study other potential damage sources for the glands in those BMT patients. The results may help with the understanding of the condition, broaden the knowledge about the SG changes outcome with standard treatment of cGVHD, and may add information for designing better therapeutic strategies for this complication.

Material and methods

The files of the BMT Unit of UNICAMP were searched for patients who had undergone allogeneic BMT between March 1994 and March 2003 and had been submitted to MSG (labial) biopsies. A total of 23 patients, identified by an exclusive patient number, who met the inclusion criteria, were selected for study. Essential requisites for inclusion in this study were the

Correspondence: Maria Letícia Cintra, Department of Pathology, Medical School, PO Box 6111, State University of Campinas, (UNICAMP), CEP (Zip code) 13081-970, Campinas - SP, Brazil. Tel.: +55 19 3788 7541. Fax: +55 19 3289 3897. E-mail: marialet@fcm.unicamp.br
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complete clinical data and follow-up, as well as available embedded tissue in the files. The conditioning regimen consisted of busulfan and cyclophosphamide, except for one patient who received fludarabine followed by total body irradiation (TBI). Bone marrow from a human leukocyte antigen (HLA)-identical sibling donor was infused on day 0, the day of the transplant [bone marrow source ($n = 14$) or peripheral blood stem cell source ($n = 9$)]. The cells were transferred without T-cell depletion or any other modification. Cyclosporin A and methotrexate were used as prophylaxis for acute GVHD in all patients. For the oral examination, they were referred to the Oral and Dental Division of Hemocentro. One patient developed grade III cutaneous, hepatic and intestinal acute GVHD. All patients (groups1 and 2) underwent systematic evaluation (GVHD screening) at day +100 (or earlier, when presenting clinical changes), consisting of physical examination, skin and lip biopsies, blood counts, liver function tests, and Schimer's testing for lacrimal function. Labial biopsy specimens were obtained from the lower inner lip, about 10 mm beneath the vermilion border, through a 4 mm-Stieffel®-punch (Stieffel, Offenbach am Main, Germany) under local anesthesia, from consenting patients. The data pertinent to prior chemotherapy, clinical examination results of the buccal mucosa, and all the prescribed medications after BMT were recorded. Fourteen of these BMT recipients developed cGVHD (group 1) and the remainig nine (group 2) were asymptomatic, had normal physical and laboratorial examination at the time of screening and, after a minimum of 60 months of follow-up, had not developed acute or chronic GVHD. In the cGVHD patients group, the median age at BMT day was 41 years (12–59) and in control group, it was 30 years (10–48). Table 1 shows the patients' data, day of first and second biopsies and follow-up. Concerning the stage of the disease, nine of 14 patients were diagnosed as having extensive cGVHD in skin, liver, and other organs. These cGVHD patients also had objective evidence of oral involvement. The remained patients presented mouth and eye involvement (three patients) and limited mouth disease (two patients), as is demonstrated in Table 2. For statistical analysis, the latter two patients' data were subsequently eliminated from the study. The diagnosis of cGVHD was made between 61 and 310 days after BMT in group 1. Those patients were treated with cyclosporin A and prednisone (1, 11) for approximately 1 year. They underwent full additional GVHD assessment at the end of treatment and were stopped immune-suppressive treatment on complete arrest or improvement of the disease to clinically acceptable performance levels. At this time, six patients had cleared all signs and symptoms of cGVHD, except for objective xerostomia, combined to xerophtalmia in four. The remaining eight patients had residual disease. Mild lichenoid/atrophic lesions in buccal mucosa were yet seen in two of the six cleared patients and in six of the remaining eight patients. Mucocele was observed in three patients (Table 2).

The second skin and MSG biopsies were obtained from 339 to 839 days after BMT. Subjective dryness, stickiness of the oral mucosa, and swallow impairment were recorded at this time. All the potential damage sources for SGs were recorded from patients' files and are displayed in Table 3. For the conventional histological examination, 6-μm paraffin-embedded sections of MSG specimens were stained with hematoxylin-eosin. The specimens were graded according to Horn et al. (12) classification (Table 4; Fig. 1). Each criterion and its

Table 1 Patients data, first and second biopsy days and follow-up span

Patient no.	*Sex*	*Age*	*Disease*	*First*	*Second*	*Follow-up (month)*
1	M	22	CML	310	626	67
2	M	15	CML	275	553	78
3	M	41	CML	61	496	47
4	F	12	CML	139	512	74
5	M	36	AML	220	502	38
6	M	46	CML	270	629	43
7	F	43	CML	214	723	103
8	F	43	CML	236	587	79
9	F	45	SAA	261	787	26 (dec)
10	F	41	CML	102	799	117
11	F	29	CML	200	534	90
12	M	59	CML	220	552	69
13	M	36	CML	198	839	43
14	M	48	MM	78	339	16 (dec)
15	M	30	CML	94		60
16	F	25	CML	110		91
17	F	34	AML	101		132
18	M	48	CML	146		73
19	F	10	SAA	141		120
20	M	36	AML	122		70
21	M	29	SAA	100		131
22	M	18	CML	133		76
23	M	32	SAA	75		127

Patient number 1–14 are cGVHD patients and 15–23 are non-cGVHD patients.
Age: age in years at BMT day; Disease: hematological disease; CML: chronic myelogenous leukemia; AML: acute myelogenous leukemia; SAA: severe aplastic anemia; MM: multiple myeloma; dec: deceased.

Table 2 cGVHD patients: buccal mucosa examination, cGVHD stage, organs involvement, treatment response, and symptoms

Patient no.	*Mucocele*	*BM 1*	*BM 2*	*cGVHD organs*	*Stage*	*Treat resp*	*Status*	*Dry*	*Stick*	*Swal*
1	No	2	0	BM,SG, S	E	Cure	Xert	No	No	No
2	No	1	Mild	SG, S	E	Cure	Xert	No	No	No
3	No	1	3	BM, SG, L	E	Improv	Xert	No	No	No
4	Yes	1	1	BM, SG, L	E	Improv	Xert	No	No	No
5	No	2	0	BM, SG	L	Improv	Xert	No	No	No
6	No	2	Mild	BM, SG	L	Improv	Xert	No	No	No
7	No	2	0	BM, SG, E	L	Cure	Xert/xerph	Yes	Yes	Yes
8	Yes	3	Mild	BM, SG, S, L, E	E	Cure	Xert/xerph	No	No	No
9	No	0	2	BM, SG, S, E, L, Lu	E	Improv	Xert/xerph	Yes	Yes	Yes
10	Yes	1	Mild	BM, SG, S, L, E	E	Improv	Xert/xerph	No	Yes	Yes
11	No	2	0	BM, SG, E	L	Improv	Xert/xerph	No	No	No
12	No	2	0	BM, SG, G, E	E	Cure	Xert/xerph	No	No	No
13	No	2	0	BM, SG, E	L	Cure	Xert/xerph	No	No	Yes
14	No	1	Mild	SG, S	E	Improv	Xert	No	No	Yes

Patient no.: BMT patient number; 1–14: cGVHD patients; 15–23: non-GVHD patients.
BM 1, buccal mucosa Horn's grades at first biopsy; BM 2, buccal mucosa Horn's grades at second biopsy or severity of lichenoid/atrophic lesions on clinical evaluation when lesions were not represented at biopsy site.
cGVHD organs: BM, buccal mucosa; SG, salivary glands; S, skin; L, liver; E, eyes; Lu, lungs; G, gastro-intestinal
Stage: E, extensive; L, limited.
Treat res: treatment response; Improv: improvement.
Status: Xert: xerostomia; Xerph: xerophtalmia; Dry: subjective dryness; Stick: subjective stickness of oral mucosa; Swal: subjective swallowing impairment.

Table 3 Potential damage sources for salivary glands and histological analysis

Patient no.	*Chronic*	*Source*	*H2*	*Hy*	*aGVHD*	*1 infl*	*2 infl*	*1 fibr*	*2 fibr*	*1st grade*	*2nd grade*
1	Yes	BM	Yes	Yes	No	Mark	Mild	Moder	Mild	3	1
2	Yes	BM	Yes	Yes	No	Mark	Moder	Moder	Mild	3	1
3	Yes	BM	Yes	Yes	No	Mild	Moder	Mild	Mark	1	4
4	Yes	BM	No	No	No	Moder	Abst	Moder	Mild	3	0
5	No	BM	Yes	Yes	No	Moder	Mild	Moder	Moder	2	1
6	Yes	BM	Yes	No	No	Moder	Moder	Moder	Moder	2	2
7	Yes	PBSC	No	Yes	No	Moder	Moder	Moder	Mark	2	4
8	Yes	PBSC	Yes	No	No	Moder	Moder	Mark	Mark	4	4
9	No	PBSC	Yes	Yes	No	Moder	Moder	Moder	Moder	2	2
10	Yes	PBSC	No	No	No	Moder	Moder	Moder	Mark	2	4
11	Yes	PBSC	No	No	No	Moder	Moder	Moder	Moder	3	2
12	Yes	PBSC	Yes	No	No	Moder	Moder	Moder	Mark	3	4
13	Yes	BM	No	Yes	No	Moder	Mild	Moder	Moder	2	1
14	Yes	PBSC	No	No	Yes	Moder	Mark	Moder	Moder	2	3
15	Yes	BM	Yes	No	No	Moder		Moder		2	
16	Yes	PBSC	No	No	No	Moder		Moder		2	
17	No	PBSC	No	No	No	Moder		Mild		1	
18	Yes	BM	No	No	No	Moder		Moder		2	
19	No	BM	No	No	No	Mild		Mild		1	
20	No	BM	No	No	No	Mild		Mild		1	
21	No	BM	No	No	No	Mild		Mild		1	
22	Yes	BM	No	No	No	Mild		Mild		1	
23	No	BM	No	No	No	Moder		Moder		2	

Patient no.: BMT patient number; 1–14: cGVHD patients; 15–23: non-GVHD patients.
Chronic: chronic myelogenous leukemia or multiple myeloma; Source: hematopoietic stem cell source; BM: bone marrow; PBSC: peripheral blood stem cell; H2: H2 receptor antagonist; Hy: anti-hypertensive drugs; 1 infl: maximum of inflammatory infiltrate observed in the first biopsy; Abst: absent; Moder: moderate; Mark: marked; 2 infl: maximum of inflammatory infiltrate observed in the second biopsy; 1 fibr: maximum of interstitial fibrosis observed in the first biopsy; 2 fibr: maximum of interstitial fibrosis observed in the second biopsy; 1 grade: Horn's grade in the first biopsy; 2 grade: Horn's grade in the second biopsy.

severity were separately recorded. Histological degree of inflammatory infiltrate and fibrosis were recorded individually for each gland represented in each biopsy specimen and the values displayed in Table 2 are the maximum values in the corresponded biopsy specimen. In order to allow an objective assessment of the salivary secretory units volume, standardized images of the PAS-stained slides were obtained using an Axiophot (Carl Zeiss, Göttinge, Germany) KS300 system. All not-superimposed microscopic fields were acquired using a 20×-objective in a sequential pattern, excluding hemorrhagic areas, histologic artifacts, incomplete fields, and the ducts. Quantification of glycoprotein was estimated using Limiar software (13). Immunohistochemistry was

Table 4 Horn's histologic grading system for salivary glands changes

Grade	*Features*
1	Mild interstitial inflammation
2	Mild acinar destruction, ductal dilation, squamous metaplasia, mucous pooling, mild fibrosis, duct cell proliferation, periductal lymphocytic infiltrate
3	Marked interstitial lymphocytic infiltrate. Diffuse destruction of ducts and acini
4	Nearly complete loss of acini, dilated ducts, interstitial fibrosis with or without inflammation

performed using the standard avidin-biotinylated peroxidase complex method. All sections were labeled with the leukocyte common antigen (LCA) primary antibody (DAKO, Carpinteria, CA, USA, code: m0701, monoclonal antibodies: PD7/26/16 and 2B11). Negative controls were performed on sections of the same cases that were similarly processed, except that primary antibodies were omitted. Unsatisfactory sections were excluded and the procedures were repeated. A known positive control for anti-LCA (normal lymph node) was included in each run. A quantification of LCA-stained cells was performed by counting the total number of positive cells from all gland surfaces using an ocular squared grid and a 40× objective. LCA is identified on B and T lymphocytes, with variable immunoreactivity for plasma cells and histiocytes. Monocytes and mast cells revealed membrane staining for LCA (14). The MSG area was taken in digitalized images of biopsies treated by immunohistochemistry (LCA). The area was viewed through an Axiophot photomicroscope (Carl Zeiss – KS300 system). Eventually, density of inflammatory cells was obtained (number of inflammatory cells/MSG area). The investigators independently and blindly viewed and calculated histopathologic variables and disagreements were re-scored for achieving a consensus. Interactive morphometry was blindly done. PAS+ volume analysis was carried out on blinded digitalized images of the glands and analyzed automatically. The following tests were applied for statistical analysis: Spearman's correlation, Pearson's correlation, Mann–Whitney test, and multiple linear stepwise regression. This study followed guidelines prescribed by the Brazilian Medical Research Centre and received Approval No 126/2004 from the Research Ethics Committee of the State University of Campinas (UNICAMP).

Results

Patients

Only five of 14 patients, who were diagnosed as cGVHD, complained of xerostomia at the end of treatment. However, clinical signs of xerostomia (absence of the sublingual salivary lake, higher adherence of the wood spatula to the jugal mucosa, dry lips and tongue snap) were still present in all patients (Table 3). Two patients died from cGVHD complications 16 and 26 months after BMT (Table 1). Patients of group 2 (control) have not presented any evidences of cGVHD

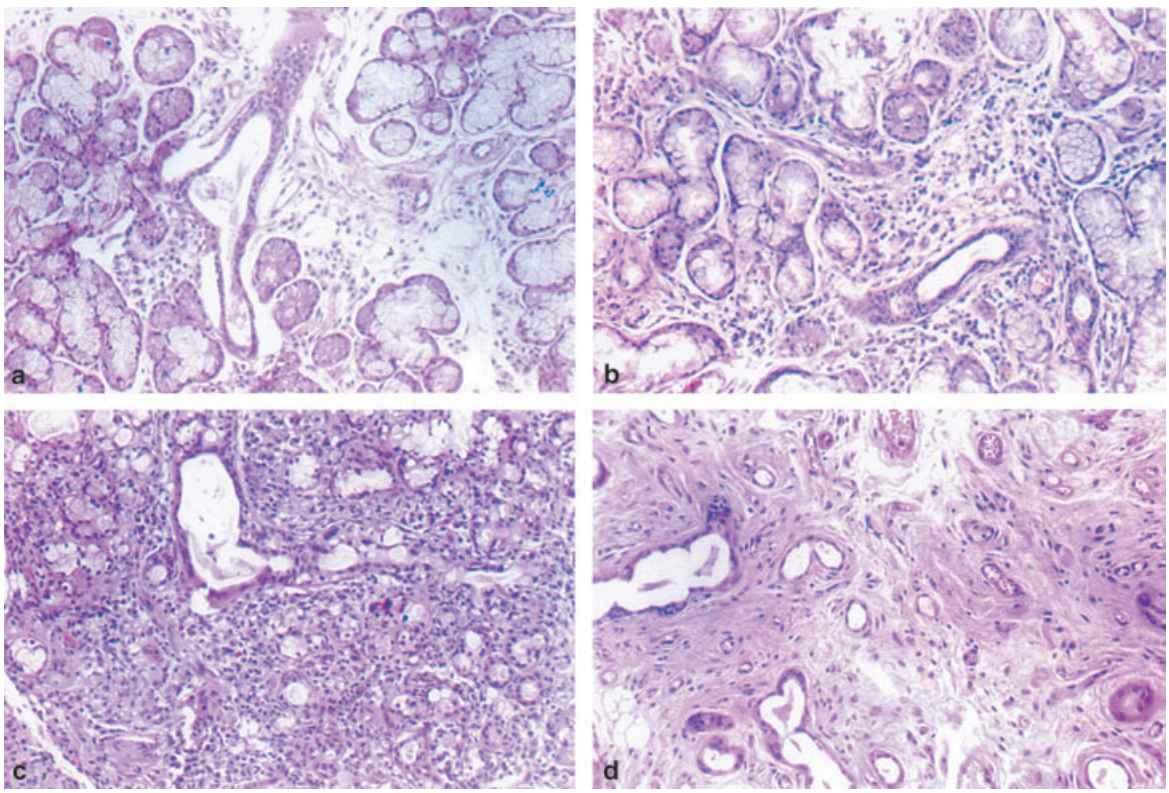

Figure 1 cGVHD (a) grade I, (b) grade II, (c) grade III, (d) grade IV (H&E, original magnification ×200).

at all routine subsequent full medical evaluation (follow-up span of 60–132 months) but presented signs of objective xerostomia as well. On statistical analysis, higher patient age was associated with worse response to treatment ($P < 0.05$). In addition to cGVHD influence, age was an important component for reduction of PAS+ (acinar) volume.

The histologic findings in MSG specimens for both cGVHD and non-cGVHD (control group) patients are shown in Table 3. Trivial inflammatory infiltrate was seen in all MSGs from patients free of cGVHD and in five of them, some degree of fibrosis.

Concerning the value of MSG histopathologic analysis for diagnosing oral GVHD, we found that the histological variables as well as Horn's grades ($P = 0.0031$) in the MSG were closely associated with the diagnosis of cGVHD. The density of inflammatory infiltrate was significantly higher in MSG of cGVHD patients (Mann-Whitney test $P = 0.029$) when compared with the control group, in both periods analyzed. Furthermore, MSG of cGVHD group had more intense fibrosis (t-test $P = 0.00148$) than those of patients without the disorder. The more intense the inflammation, the more intense was the fibrosis (Spearman correlation). In a multivariate regression, cGVHD was shown the variable that determined fibrosis of SGs. MSG of patients with cGVHD showed significantly less PAS- positive (acinar) volume when compared with control group patients (t-test $P = 0.01847$). A correlation with the day of the biopsy was observed (Pearson's correlation) because the diagnosis of cGVHD had mostly been assessed in a later period, comparatively to the unique day 100 screening biopsies of the control group. There were no differences in sex and type of transplantation regarding inflammatory index, fibrosis, or PAS+ (acinar) volume. To analyze potential damage sources else for SGs, we observed that many different hematological diseases (and correlated treatment) resulted, in our study, in very small groups. We had to join some in order to get a reasonable test power. The criteria used for joining was based on the disease treatment. Patients with chronic diseases (chronic myelogenous leukemia and multiple myeloma) were supposed to receive a greater 'drug burden' than patients with shorter illness (acute leukemias) or aplastic anemia (no chemotherapy). Therefore, the diseases were divided in two groups: those with prolonged course (chronic myelogenous leukemia and multiple myeloma) and those with a shorter course (acute leukemias) together with aplastic anemia. Significant correlation was obtained between PAS+ (acinar) volume and chronic basic disease (t-test $P = 0.01428$). Use of antihypertensive drugs was highly significant (t-test $P = 0.00672$) for PAS+ volume decrease. In order to overcome confounders, we run a multiple linear stepwise regression and the results were the same.

Use of antihypertensive drugs and chronic basic disease were correlated to diminished volume of PAS (acinar) material. Patients with cGVHD had taken much more drugs than patients without. There was a clear-cut correlation between the number of drugs used after BMT and the degree of inflammation. The possible influence of the hematological disease, use of drugs, and age, in MSG fibrosis was tested in a multivariate regression analysis. Only use of H2 receptor antagonists ($P = 0.037$) and long lasting hematological diseases ($P = 0.04$) were independent factors for the degree of fibrosis in MSG. Concerning treatment results, cGVHD glands still showed significantly less acinar PAS+ volume in the second biopsy when compared with MSGs of the control group at day 100+ ($P = 0.014$). Comparing the PAS positive dyestuff before and after treatment, there was no significant difference ($P = 0.17009$). No difference was seen in leukocyte index ($P = 0.10498$) or in fibrosis ($P = 1.0$). No difference was obtained in cGVHD Horn's grading ($P = 0.88372$) before and after treatment.

Discussion

Chronic GVHD is one of the most serious complications after allogeneic BMT and SGs are a known major target (15, 16). The salivary changes are expressed by a reduction in related functions, such as anti-infectious activity, protection against mechanical and chemical injuries, assistance in controlling periodontal disease and cavities, contribution to verbal communication, nutrition and soft tissue repair (17, 18). In fact, salivary hypofunction increases the risk for multiple complications, including mucositis and dysgeusia, as well as oral and systemic infection (19). Systematic oral follow-up of patients undergoing BMT has been carried out at the Oral and Dental Outpatient Center of the BMT Unit at UNICAMP, with the purpose of: (i) prior to BMT, to screen and control focuses of infectious activity that could enhance patient morbidity during the aplasia period after the conditioning regimen; (ii) to detect and control, in the long-term survivors, oral manifestations of cGVHD and its multiple complications. During the past 11 years, we have observed that patients assisted at our Unit, whether developing cGVHD or not, presented: (i) xerostomia; (ii) generalized dental tenderness that has been, in the great majority of cases, responsive to topical fluoride application; (iii) higher frequency and severity of cavities, sometimes similar to radiation-associated cavities, as well as precocious infiltration of dental restorations and periodontal disease. Dental restorations were impaired by mouth dryness, even with the use of glass ionomer, leading to dental decay. Fungal (candidiasis) and viral (herpes, HPV) infection frequently ensued after total dental loss. Patients with objective signs of xerostomia (absence of the salivary pooling in the floor of the mouth, dry and sticky buccal mucosa with higher adherence of the wood spatula to the jugal mucosa, dry lips and tongue snap) seemed to get used to their condition since, on several occasions, they denied subjective sensation of dry mouth (as well as spitting and swallowing impairment). For these reasons, it was difficult to establish clinical parameters for cGVHD treatment response. In fact, only five of 14 cGVHD patients in this study, independently of the histological degree of MSG destruction after the end of

treatment, had complained of xerostomia. Therefore, according to our observations subjective xerostomia had low specificity in the diagnosis of cGVHD activity and might also be caused by drugs. These findings are in keeping with Peterson's experience (19) who emphasizes that symptom of xerostomia does not necessarily correlate with the signs of SG hypofunction. An association between GVHD and the clinical impression of xerostomia was demonstrated in Schubert et al. (7) study. Nagler et al. (20) found no hyposalivation in patients who underwent A-BMT but did not develop cGVHD, as compared with normal individuals. They reported a direct correlation between the degree of hyposalivation and the severity of the cGVHD. Based on several earlier studies of MSG histopahology, starting with the work of Sale et al. (8), the value of oral biopsies, including MSG assessments for diagnosing oral GVHD has been established. Therefore, in order to clarify SGs involvement in our patients, we chose to evaluate histological alterations in MSG, which are very simple to obtain and can reflect both the alterations in major SG and the degree of cGVHD involvement in other organs (7, 21).

The more precise the diagnosis of cGVHD is, the greater the assistance in predicting the severity and, thus, the outcome of the disease, as chance of remission and risk of infection (22). Our results, together with those achieved by others (7, 8, 22), suggest that a pathological examination of the MSGs is useful in grading as well as in the diagnosis of cGVHD. Our histological findings in the MSG and Horn's grades were closely associated with clinical diagnosis of cGVHD. We observed that the density of inflammatory cells and fibrosis played a significant role in clinical diagnosis of cGVHD. Non-cGVHD patients showed less frequent abnormalities in MSG. Nakamura et al. (21) reported that, statistically, the presence of diffuse and periductal lymphocytic infiltration in MSG shows a relationship to the diagnosis of cGVHD. Levy et al. (23) reported an inverse correlation between the mean parotid salivary flow rate and the degree of fibrosis seen in the histopathological evaluation of cGVHD mice. They have found profound differences in inflammatory infiltration and fibrosis in the cGVHD group in contrast to those observed in both the syngeneic transplanted group and the normal (non-transplanted mice) group. Concerning the role of other potential sources for SGs injury, we have already found interstitial fibrosis of MSGs at diagnosis, in cGVHD patients, which means part of the damage probably started earlier. Furthermore, as fibrosis was also seen in five of nine control group patients, it could not be ascribed purely to cGVHD. The list of possible causes of chronic sialoadenitis in BMT recipients includes drugs, radiation, and acute GVHD, among others. Ionizing radiation can cause permanent damage to SGs, which is manifested as acinar cell destruction with subsequent atrophy and fibrosis of the glands (24). Although, only one patient received TBI. He developed severe acute GVHD and died from complications of cGVHD, 16 months after BMT. With respect to the role of drugs on MSG damage, is well known that frequent symptoms in patients undergoing chemotherapy are mouth dryness. This may be due to a reduction in the salivary flow from the MSGs (25). Lockhart and Sonis (26) studied the histologic changes that occurred in SGs, at autopsy, as a result of chemotherapy for cancer. Ductal dilation, cyst formation, some acinar degeneration, and infiltration of inflammatory cells were seen. The effect of previous chemotherapy combined with transplant conditioning regimens has potential for acinar damage and, for some patients there may not be full recovery. In the chronic leukemia patients that are supposed to receive long lasting chemotherapy treatment, we have observed statistically diminished PAS (acinar) volume. It is also interesting that patients salivary flow rates may decrease simply in proportion to the number of prescription drugs they are taking (27). We observed a clear-cut correlation between the number of drugs taken after BMT and the degree of inflammation. Patients with cGVHD were consuming much more drugs than patients without. A wide range of drugs can be xerogenic, as antihypertensive drugs and H2-receptor antagonist (27), which were prescribed to some of our patients before and after BMT. Indeed, there was statistical correlation between the use of anti-hypertensive drugs and diminished PAS (acinar) volume. Additionally, a multivariate regression analysis of the possible influence of the hematological disease, use of drugs, and age revealed that the use of H2 receptor antagonists and long lasting basic disease were independent factors for the degree of fibrosis in the SGs. In sum, while the exact effects of all various single or multiple drug chemotherapy regimens on SGs is far from clear, our results do support the fact there is some degree of permanent damage. With respect to acinar atrophy, our results revealed that MSG of patients with cGVHD showed significantly less PAS-positive acinar volume when compared with control group patients ($P = 0.01$) at the diagnosis of cGVHD as well as at the end of the treatment. MSG acinar regeneration did not occur at all in eight of 14 patients. It was demonstrated that, in cGVHD patients, the reduction in flow rates correlates with the degree of SG destruction observed in the pathological slides (23). Nagler and Nagler (17) found that SG involvement in cGVHD occurs very rapidly, is severe, and recovery does not occur during the following year in which patients are monitored. In our study, the inflammatory infiltrate density that was seen at the end of therapy was greater than in the control group. No difference was seen in PAS+ (acinar) volume, leukocyte index, fibrosis, or cGVHD Horn's grading between the first and post-treatment biopsies. The putative explanation is that some patients improved but others did not. LCA-positive cells were seen among acini and ductal epithelial cells in both, first and second (post-treatment) biopsy specimens. Previous works have demonstrated that cGVHD resembles an autoimmune-like disorder. Tissues are damaged via the cytotoxicity rendered by the infiltrating donor graft T cells. It also involves auto reactive lymphocytes (28–30). The complex pathophysiology of cGVHD fundamentally

depends on interactions between antigen-presenting cells (APCs) of the recipient and mature T-cells of the donor, HLA-up regulation, mononuclear infiltration, and cytokine dysregulation (5, 31, 32). Sale et al. (8) reported that in the majority of tissue involvement by GVHD, epithelial cells are the focus of lymphocyte attack. Initial attack to MSG probably involves ductal attack by lymphocytes and acinar damage is actually retrograde and subsequent to ductal dysfunction. The targets and focus of the inflammatory infiltrate may be, at least in part, because of damage caused by duct dysfunction leading to acinar breakdown. Progressively, there is acinar cell death and eventually destruction of the secreting units and replacement by fibrosis or simple loss of units. In fact, the production of mucoceles, supposedly because of ductal inflammation and damage (7), were noted in three of 14 cGVHD patients and in none in the control group. Takahashi et al. (33) studied regeneration of doubly duct-ligated atrophic rat parotid gland in which no acinar cells remained. It was found that the acinar cell precursors first arose from residual ducts but subsequent growth was largely dependent on the progressive differentiation and proliferation of these newly formed cells. The release of duct obstruction resulted in a rapid drop in duct cell proliferation and the appearance of cells that progressively differentiate into mature acinar cells. They speculated that such differentiating cells are the ones that continued to die in the duct-obstructed atrophic gland. In cGVHD, as the gland remains atrophic in the presence of lymphocyte infiltration, the proliferation must be abortive, because new cell formation is presumably balanced by ongoing apoptotic cell death. Our results suggest that persistent xerostomia after cGVHD treatment is because of maintenance of lymphocytic infiltrate and consequent absence of SG secretor unit's recovery. Indeed, at the end of treatment, six of 14 cGVHD patients have cleared all signs and symptoms of cGVHD except for the MSG–and they were successfully taken off of systemic therapy despite this MSG cGVHD activity (two of them also presenting buccal mucosa lichenoid/atrophic lesions). They have been managed with dental decay treatment and prevention (maintenance of optimal oral hygiene, low sucrose-containing diets, daily concentrated topical fluoridre administrations), candidiasis treatment, and salivary substitutes, as classically recommended (19).

In conclusion, our results, as well as others' (7, 8) support the use of MSG biopsies to diagnosis cGVHD and can further promote the utility for the assessment of the response to treatment. The effects of previous chemotherapy, combined with transplant conditioning regimens, and medications received after BMT must be considered.

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