EXPRESSION OF CD80/CD86-CD28 COSTIMULATORY MOLECULES BY PERIPHERAL BLOOD MONONUCLEAR CELLS AND SALIVARY GLANDS OF PATIENTS WITH SJÖGREN'S SYNDROME

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Abstract: *Objective.* To investigate the expression of costimulatory molecules CD80, CD86, and CD28 on peripheral blood monouclear cells (PBMC) and in salivary gland (SG) biopsy tissues of patients with Sjögren's syndrome (SS).
Methods. Monoclolal antibodies against CD80, CD86, and CD28 were used for flow cytometry and immunohistochemistry. PBMC CD80, CD86, and CD28 were studied in 21 patients with primary SS and 22 healthy controls, and biopsy tissues were studied in 12 patients with primary SS and 5 with secondary SS (3 with systemic lupus erythematosus and 2 with rheumatoid arthritis).
Results. CD28 expression on $CD4^+$ and $CD8^+$ T cells was lower in SS patients than in normal controls. CD80 and CD86 were expressed on infiltrating mononuclear cells (MNC) in SG biopsy specimens; the majority were adjacent to acini and ducts, with a few MNC in large lymphoid aggregates. Staining for CD80 and CD86 also revealed positive acinar and ductal endothelial cells in SG biopsy specimens.
Conclusion. A costimulatory signal CD80 and CD86 may be required for the induction of lymphoepithelial lesions of SG, but not for progression of SS. CD80 and/or CD86 expressed on acinar and ductal endothelial cells is associated with activation of T cell in SG tissues of SS patients. (奈医誌. J. Nara Med. Ass. **51**, 381～393, 2000)

Key words: CD80, CD86, CD28, salivary gland, Sjögren's syndrome

INTRODUCTION

Sjögren's syndrome (SS) is a systemic autoimmune disorder characterized by keratoconjunctivitis sicca and xerostomia resulting from lymphocytic infiltration of lacrimal and salivary glands (SG)[1]. Infiltrating T and B cells are activated, and often form a germinal center, which is considered a major site for production of autoantibodies. Sequence analysis of the B cell immunoglobulin variable region from lymphocytes obtained from SG have suggested that B cell differentiation may occur within the gland[2]. To mount an effective response, antigen-specific T cells require at least 2 distinct signals on contact with antigen-presenting cells (APC). The first signal involves the T cell receptor (TCR) and MHC class II molecules. Recognition of the first signal leads to up-regulation of CD40L on T helper cells. CD40L interacts with its counterreceptor, CD40, on B cells and other APC, inducing up-regulation of another set of costimulatory (CD80 and CD86) and adhesion molecules, as well as cytokine production by APC[3-7]. To induce complete T cell activation, costimulatory signals between CD28 on the T cell and CD80 or CD86 on APCare essential[8]. Without these costimulatory signals, T and B

cells are rendered inactive by anergy or deletion[8].

CD28 is expressed on the surface of most $CD4^+$ T cells and about 50% of $CD8^+$ T cells. CD28 has a lower avidity for CD80 and CD86 than cytotoxic lymphocyte-associated molecule-4 (CTLA-4), which is a downregulatory molecule in T cell activation[9,10]. The major role of CD28 signaling is to prevent apoptosis and sustain proliferation of T cells[11].

Recent studies found that CD80/CD86 and CD28/CTLA-4 interactions play important roles in the pathogenesis of autoimmune diseases. Anti-human CTLA-4 treatment was reported to block the development or progression of disease in animal models of lupus nephritis[12], experimental allergic encephalomyelitis[13,14], and type 1 diabetes[15].

Here we report analysis of the expression of CD80 and CD86 on peripheral blood B lymphocytes and CD28 on peripheral blood T lymphocytes from SS patients. We also sought to determine the cellular expression and distribution of surface CD80, CD86, and CD28 in biopsied SG tissue derived from SS patients. Our findings suggest that a costimulatory signal through CD80 and CD86 is essential for the early phase of development of lymphoid lesions of SG.

MATERIALS AND METHODS

Patients and healthy controls. SS was defined by the previously established European classification criteria[16]. Samples of peripheral blood were obtained from 21 patients with primary SS followed at Nara Medical University. All were women with a mean age of 58 years (range 44 to 73 years). Blood samples were also obtained from 22 healthy controls; women with a mean age of 50 years (range 41 to 67 years). Following informed consent, labial minor SG biopsy samples were obtained from 12 patients with primary SS and 5 with secondary SS (3 with systemic lupus erythematosus (SLE) and 2 with rheumatoid arthritis (RA)); all were women with a mean age of 52 years (range 24 to 71 years). Patient profiles are shown in Table 1.

Antibodies. The following monoclonal antibodies were used to study peripheral blood mononuclear cells (PBMC) for CD80 and CD86: fluorescein isothiocyanate (FITC)-labeled anti-CD80 (DAL1, murine IgG1; Caltag, Burligame, CA) and FITC-anti-CD86 (BU63, murine IgG1; Ancell, Bayport, MN); phycoerythrin (PE)-labeled anti-CD19 (4G7, murine IgG1; Becton Dickinson, San Jose, CA) and for CD28: FITC-anti-CD28 (KOLT-2, murine IgG1; Nichirei, Tokyo, Japan); PE-anti-CD4 (SK3, murine IgG1; Becton Dickinson); and PE-anti-CD8 (SK1, murine IgG1; Becton Dickinson). The following monoclonal antibodies were used for immunohistochemistry: anti-CD80 (L307.4, murine IgG1; Becton Dickinson); anti-CD86 (FUN-1, murine IgG1; Pharmingen, San Diego, CA); anti-CD28 (CD28.2, murine IgG1; Pharmingen); anti-Leucocyte Common Antigen (LCA) (T29/33, murine IgG1; Dako, Glostrup, Denmark); and anti-CD4 (SK3, murine IgG1; Becton Dicknson).

Flow cytometry analysis. For two-color immunofluorescence experiments, antibodies were added to whole blood for 30 minutes at 4℃. Erythrocytes were lysed with lysing solution (0.154M NH_4Cl, 0.01M $KHCO_3$, and 0.088 mM EDTA). Leukocytes were pelleted and washed with PBS-BSA buffer[17]. The sample was resuspended in FACS Flow (Becton Dickinson), and cells were analyzed using a FACScan (Becton Dickinson).

Biopsy of labial salivary glands. Under local anesthesia, biopsy of the minor labial SG was

Table 1. Demographics and clinical characteristics of patients with primary and secondary Sjögren's syndrome

Patient	Age	Complication	SG biopsy focus score	Ro/SS-A	La/SS-B
1	49		1	−	−
2	50	SLE	1	+	+
3	68		1	−	−
4	71		1	+	−
5	24	SLE	4	+	+
6	44		4	−	−
7	45	SLE	3	+	−
8	47		9	+	−
9	49		2	+	+
10	51		2	−	−
11	52		8	−	−
12	53		5	−	−
13	56	RA	6	+	−
14	57		3	+	−
15	57		9	+	+
16	58	RA	5	+	−
17	60		13	+	−

SG, salivary glands; Ro/SS-A, antibodies to Ro/SS-antigens; La/SS-B, antibodies to La/SS-B antigens; SLE, systemic lupus erytematosus; RA, rheumatoid arthritis

performed from the mucosa of the lower lip. Tissues were frozen in liquid nitrogen and stored at -80℃ until use.

Immunohistochemistry. Frozen tissue sections were air dried at room temperature for 10 minutes and fixed in 4% paraformaldehyde phosphate buffer for 30 minutes. Nonspecific antibody binding and endogenous peroxidase activity were blocked by preincubating specimens with 10% rabbit serum (Nichirei) and 0.6% H_2O_2/MeOH. Specimens were incubated with primary antibodies at 1 : 50 dilution at 37℃ in a humidified chamber for 120 minutes. These were followed by sequential incubations with biotin-conjugated rabbit anti-mouse lg for 90 minutes and peroxidase-labeled streptavidin for 30 minutes (both from Nichirei). All incubations were followed by three 5-minute washes in phosphate-buffered saline (PBS). Bound peroxidase was developed with 0.05% diamino-benzidine tetrahydrochloride (Wako Pure Chemicals, Osaka, Japan) -0.03% H_2O_2 in Tris buffered saline. Biopsy sections were counterstained with hematoxylin.

Staining of biopsy specimens with each antibody was expressed as the percentage of positive acini, ducts, or infiltrating mononuclear cells (MNC) by light microscopy at ×400 magnification. Positive staining of acini or ducts had to include acinar or ductal epithelial cells. Positive cells in each section were counted field-by-field by 2 independent observers (YU, TF) who were unaware of the diagnosis and antibody. The severity of labial SG lesions was defined by the number of focal lesions: group 1, one focus; group 2, ≧2 foci. One focus was defined as an aggregate of at least 50 MNC, and the number of foci per 4 mm^2 of glandular tissue was evaluated[18]. Moreover, each aggregation of lymphocytes was classified into three classes: class 1, a cluster of 20 to 100 lymphocytes; class 2, a cluster of 101 to 1000 lymphocytes; and

class 3, a cluster of more than 1000 lymphocytes. The correlation between the percentage of positive stained cells and the number of lymphocytes in each cluster was examined.

Statistical analysis. Means±SEM are used throughout the text, and differences are compared using the Mann Whitney *U* test. P values <0.05 were considered significant.

RESULTS

CD80, CD86, and CD28 positive on PBMC in SS. We analyzed CD80 and CD86 expressing lymphocytes in peripheral blood from 21 SS patients and 22 healthy controls. There was no difference in the percentage of $CD80^+CD19^+$ cells or $CD86^+CD19^+$ cells between SS patients and healthy controls (1.4±0.2 vs 1.7±0.2 and 4.7±1.1 vs 3.3±0.3%). The percentages of $CD28^+$ $CD4^+$ cells and $CD28^+CD8^+$cells from SS patients were significantly lower compared to healthy controls (88±2 vs 93±2; p=0.008 and 34±3 vs 49±3%; p=0.006)(Fig. 1).

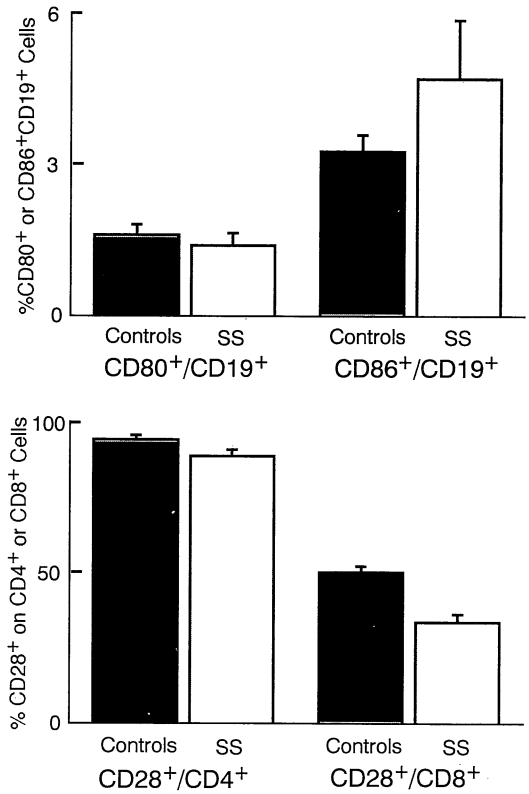

Fig. 1. Expression (mean±SEM) of CD80 or 86 in $CD19^+$ B cells, and CD28 in $CD4^+$ or $CD8^+$ T cells in peripheral blood. There was no difference in the percentage of $CD80^+/CD19^+$ and $CD86^+/CD19^+$ cells between SS patients and healthy controls (1.4±0.2 vs 1.7±0.2 and 4.7±1.1 vs 3.3±0.3%). The percentages of $CD28^+/CD4^+$ and $CD28^+/CD8^+$ cells from SS patients were lower than those in halthy controls (88±2 vs 93±2, p=0.008 and 34±3 vs 49 ±3%, p=0.006).

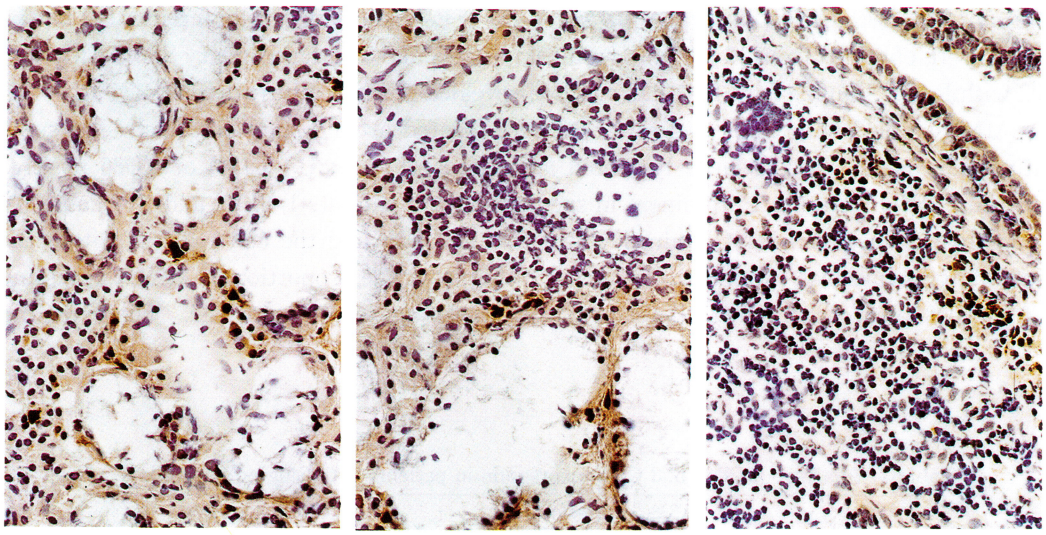

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Fig. 2. Expression of CD80 in labial salivary glands (SG) from SS patients. A. Representative sample of CD80 expreession on infiltrating mononuclear cells (MNC) in class 1. B. Representative sample of CD80 expression on infiltrating MNC in class 2. C. Representative sample of CD80 expression on infiltrating MNC in class 3. (Original magnification ×400.)

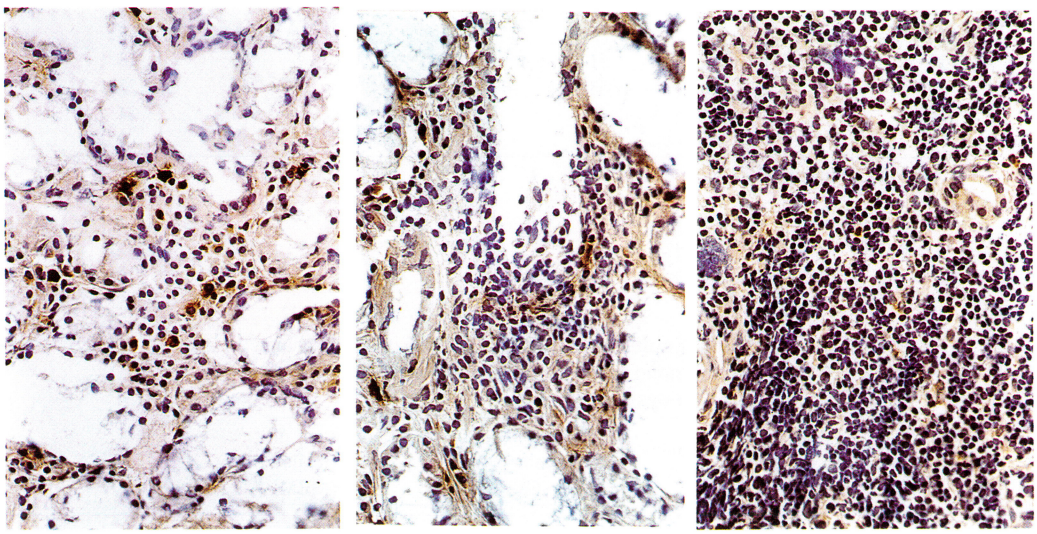

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Fig. 3. Expression of CD86 in labial SG from SS patients. A. Representative sample of CD86 expression on infiltrating MNC in class 1. B. Representative sample of CD86 expression on infiltrating MNC in class 2. C. Representative sample of CD86 expression on infiltrating MNC in class 3. (Original magnification × 400.)

Expression of CD80, CD86, and CD28 in minor SG biopsy specimens. Labial minor SG biopsy samples were obtained from 12 patients with primary SS and 5 with secondary SS (3 with SLE and 2 with RA). Staining for CD80 and/or CD86 revealed positive infiltrating MNC (Table 2). Most MNC stained with CD80 and/or CD86 were located around acini and ducts, but some positive cells were noted in large lymphoid aggregates (Fig. 2, 3). CD80 or CD86 positive infiltrating MNC were most prominent in specimens from patients 1, 8, 16, and 17 (Table 3).

A correlation between MNC stained with CD80 or CD86 and the cell number within the lymphoid aggregate was examined. As shown in Figure 4, the proportion of MNC expressing CD80 or CD86 decreased as the number of MNC in the aggregate increased. There was no

Table 2. Analysis of CD80 and CD86 expression in patients with Sjögren's syndrome

Patient	SG biopsy focus score	CD80 expression			CD86 expression		
		MNC/mm2	acinus(%)	duct(%)	MNC/mm2	acinus(%)	duct(%
1	1	36	21	0	44	18	8
2	1	7	18	11	10	17	17
3	1	26	7	78	27	11	46
4	1	3	7	32	5	12	49
5	4	45	13	46	66	32	46
6	4	22	23	64	12	15	50
7	3	5	29	4	4	18	4
8	9	47	34	20	51	34	12
9	2	9	11	11	12	9	10
10	2	31	18	50	18	41	44
11	8	25	37	60	46	37	67
12	5	8	37	8	12	18	17
13	6	5	20	9	10	21	6
14	3	11	11	27	12	12	18
15	9	41	53	20	50	52	27
16	5	40	22	29	47	15	24
17	13	45	10	0	46	2	0

SG, salivary glands; MNC, mononuclear cells

Table 3. Expression of CD80 and CD86 on infiltrating mono nuclear cells from salivary glands biopsy specimens

Class	Number of foci	Percentage of $CD80^+$ cells (mean±SEM)	Number of foci	Percentage of $CD86^+$ cells (mean±SEM)
1	19	14.6±1.8	20	11.4±1.1
2	10	4.5±1.4	13	5.1±1.1
3	4	0.3±0.2	4	0.4±0.2

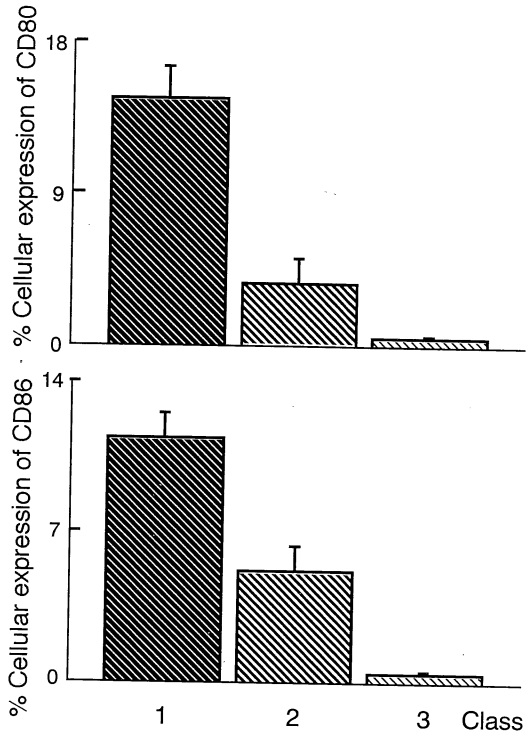

Fig. 4. Expression (mean±SEM) of CD80 and CD86 on infiltrating MNC in minor SG biopsy specimens from SS patients. The proportion of MNC expressing CD80 or CD86 decreased as the number of MNC in lymphoid aggregates increased. These differences were significant between classes ($p<0.01$), except for the difference between class 2 and 3 for CD80. There were no differences in the percentages of $CD80^+$ and $CD86^+$ positive MNC within the same class.

difference between the percentage of MNC stained with CD80 versus CD86 within the same class. The number of surviving acini decreased in proportion to the severity of the labial SG lesions. In order to examine a correlation between CD80 or CD86 expression and the severity of labial SG lesions, the number of $CD80^+$ or $CD86^+$ MNC/mm^2, the ratio of $CD80^+$ or $CD86^+$ MNC to LCA^+ cells, and the ratio of $CD80^+$ or $CD86^+$ MNC to the number of acini were analyzed. There was no difference in any of these ratios between the two groups (Fig. 5). Staining for CD80 or CD86 was positive both on acinar epithelial cells and on ductal epithelial cells, as well as on infiltrating MNC (Fig. 6). As shown in Table 2, the percentage of $CD80^+$

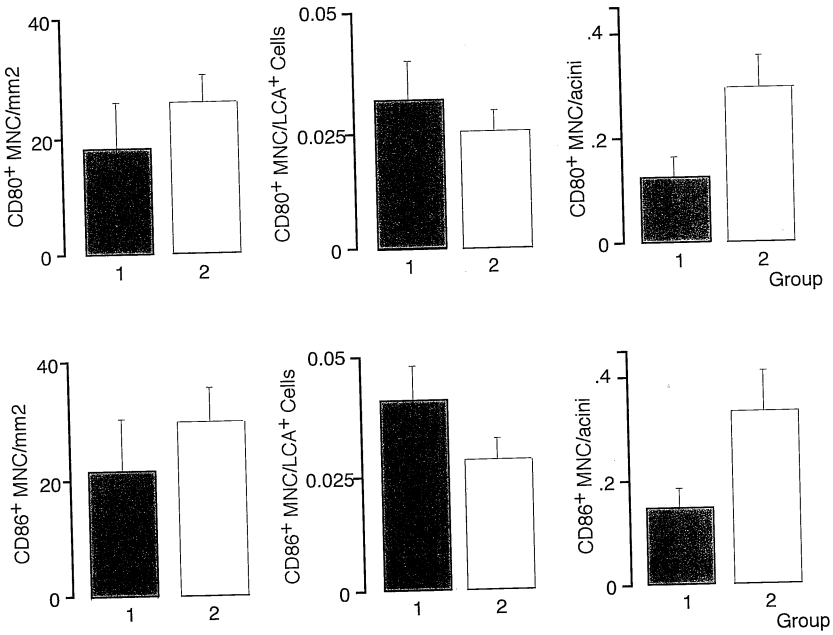

Fig. 5. CD80+ or CD86+ MNC/mm², CD80+ or CD86+ MNC/LCA+ cells, and CD80+ or CD86+ MNC/acini (mean±SEM) in minor SG biopsy specimens from SS patients. There were no differences in these ratios between the two groups of minor SG biopsy specimens.

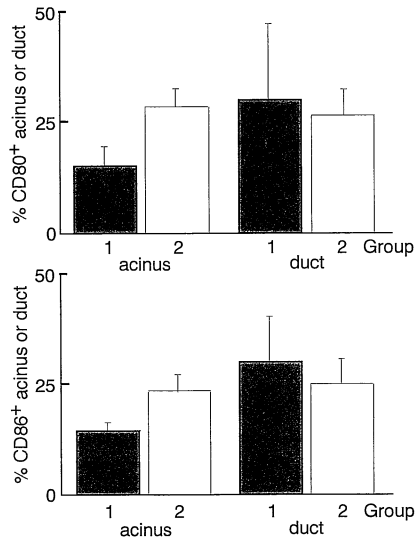

Fig. 7. Expression (mean±SEM) of CD80 and CD86 in acini and ducts in minor SG biopsy specimens from SS patients. There was no difference in expression of between the two groups.

acini was 7 to 53% and that of $CD80^+$ ducts was 0 to 78%. The percentage of $CD86^+$ acini was 2 to 52% and that of $CD86^+$ ducts was 0 to 67%. There was no difference in the percentage of CD80 or CD86 expression on acini or ducts between the two groups (Fig. 7). Staining for CD28 was positive on infiltrating MNC (Fig. 8), and 50 to 80% of infiltrating $CD4^+$ T cells were positive for CD28.

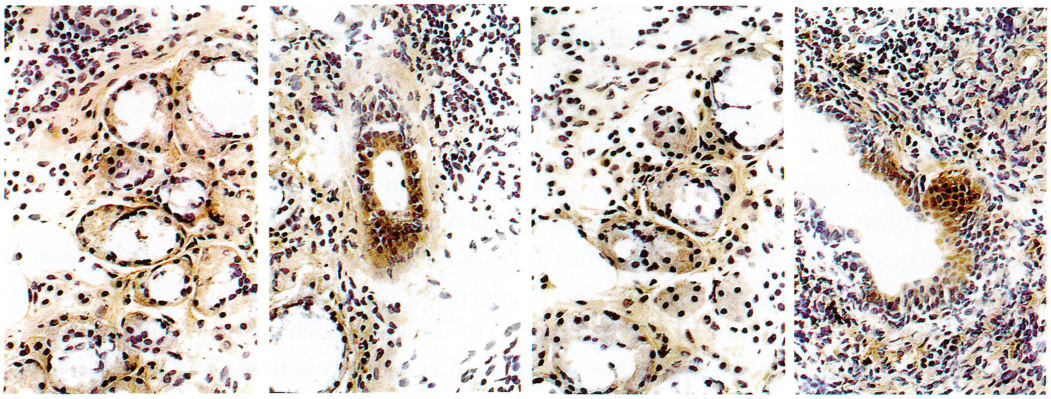

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Fig. 6. Expression of CD80 and CD86 on acinar or ductal epithelial cells in labial SG from SS patients. A. Representative sample of CD80 expression on acinar epithelial cells. B. Representative sample of CD80 expression on ductal epithelial cells. C. Representative sample of CD86 expression on acinar epithelial cells. D. Representative sample of CD86 expression on ductal epithelial cells. (Original magnification ×400.)

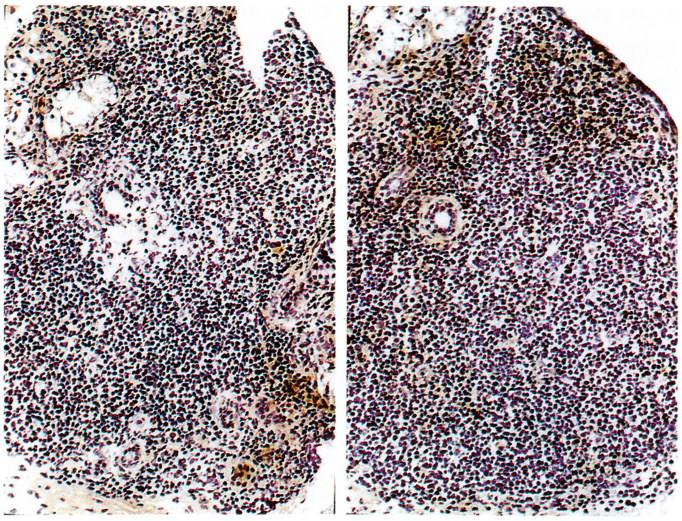

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Fig. 8. Expression of CD28, CD4, and CD19 in SG from SS patients. A. Representative sample of CD28 expression on infiltrating MNC. B. Representative sample of CD4 expression on infiltrating MNC. (Original magnification ×200.)

DISCUSSION

Our data showed no difference in the percentage of $CD80^+$ or $CD86^+$ B cells between SS patients and healthy controls. Folzenlogen et al. demonstrated increased CD86 expression on resting and activated peripheral blood B cells in SLE patients and a similar but less marked increase in CD80 expression[19]. It is not known whether increased expression of CD80 or CD86 on PBMC is causal to or simply a reflection of the disease process in SLE. Although increased CD80 or CD86 expression on MNC in synovial fluid, cerebrospinal, and other tissues from autoimmune dineases has been observed, increased expression of CD80 or CD86 on PBMC has only been reported in SLE[20-22]. Therefore, the number of resting peripheral blood B cells expressing CD80 or CD86 may not reflect the development of lymphoepithelial lesions in SS. Increased numbers of B cells expressing B7/BB1, which includes CD74 as well as CD80[23], were reported in autoimmune thyroid disease[24], and we found a similar result using anti-B7/BB1 antibody on PBMC in our SS patients (data not shown).

We demonstrated that the percentage of $CD28^+CCD4^+$ cells and $CD28^+CD8^+$ cells from SS patients was lower than in healthy controls. A decrease in circulating $CD28^+$ cells has been reported in patients with RA or SLE[25,26]. $CD28^-CD4^+$ T cells, which frequently undergo clonal expansion in RA patients, expressed higher amounts of bcl-2 protein than $CD28^+CD4^+$ T cells[27]. $CD28^-CD4^+$ T cell clones did not express messenger RNA for CD40 ligand and therefore do not have the ability to provide B cell help[28]. $CD28^-CD4^+$ T cells have a role in the extraarticular manifestations of RA[29]. Anti-CD3-induced apoptosis of $CD28^+$ T cells was significantly accelerated in SLE, whereas apoptosis of $CD28^-$ T cells did not occur in either SLE or normal controls[26]. Decreased expression of CD28 on PBMC from SS patients therefore indicates that $CD28^-CD4^+$ T cell-mediated cytotoxicity may be important in SG involvement.

CD80 and/or CD86 proteins were expressed on acinar and ductal epithelial cells, as well as infiltrating MNC in SG tissues from patients with SS. In lesional skin from patients with SLE, subacute cutaneous lupus erythematosus, or chronic discoid lupus erythematosus, dermal and epidermal nonprofessional APC expressed CD80 and CD86[30]. SG epithelial cell lines derived from SS patients strongly express CD80, but less so CD86 ; INF γ treatment of those cell lines resulted in the induction of class II MHC and up-regulation of both CD80 and CD86[31]. Several molecules have been proposed as candidate autoantigens for development of SS. Matsumoto et al. demonstrated that alpha-amylase functions as a SG-specific T cell epitope and induces autoimmunity in SS[32]. Haneji et al. proposed α-fodrin as an autoantigen in primary SS[33]. The expression of CD80 and/or CD86 and MHC proteins in SG epithelia from SS patients after activation with INF γ suggests their potential to function as APC in the development of lymphoepithelial lesions. Combined with the studies mentioned above, our results indicate that specific expression of CD80 and CD86 costimulatory molecules on acinar and ductal epithelial cells may be important for activation of T cells in SG tissues.

We found no difference between the percentage of $CD80^+$ and $CD86^+$ MNC in minor SG biopsy specimens. $CD4^+$ T helper (Th) cells, upon antigenic stimulation, differentiate into Th1 and Th2 subsets, each producing its own set of cytokines and mediating separate effector functions[34]. Kuchroo et al. showed that CD80 and CD86 costimulatory molecules differentially activated Th1 and Th2 developmental pathways[35]. On the other hand, Levine et al. reported

no intrinsic differences between CD80 and CD86 in their ability to co-stimulate T cells[36]. Th1 cytokines are essential in the induction and/or maintenance of SS, while Th2 cytokines are involved in the progression of disease, especially through local B cell activation[37]. Similarly, the development of experimental SLE in mice involves two stages : increased production of Th1 -type cytokines, followed by induction of Th2-type cytokines[38]. Thus, the conversion of Th1 to Th2 cytokine production by T cells in SS and SLE seems to reflect disease progression. In our study, however, differential expression of CD80 or CD86 by professional APC was not found. The relevance of $CD80^+$ or $CD86^+$ MNC to disease progression in SS remains unclear.

We found that the majority of infiltrating MNC expressing CD80 or CD86 were located around acini and ducts in SG biopsy specimens. A few MNC in large lymphoid aggregates were positive for CD80 or CD86. Moreover, the proportion of MNC which expressed CD80 or CD86 decreased, as the number of MNC in lymphoid aggregates increased. Thus, a costimulatory signal through CD80 or CD86 may be required for the induction of lymphoepithelial lesions of SG.

In conclusion, the expression of costimulatory molecules on SG epithelia suggests their potential to function as APC to further activate T cells in the development of lymphoepithelial lesions in SG tissues, and these costimulatory signals through CD80 and/or CD86 may be essential for the early development of the characterstic lymphoid lesions of SG in SS patients. Decreased expression of CD28 on PBMC indicates that $CD28^-CD4^+$ T cell-mediated cytotoxicity has an important role in the pathogenesis of SS.

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