qualitative data. Receiver operating characteristic (ROC) curve analysis was used to analyze cut-off levels of HBcrAg concentration for prospective recurrence of hepatitis. Statistical analyses were performed using the SPSS 14.0 J statistical software package (SPSS, Chicago, IL, USA), and a *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

TWENTY (59%) OF the 34 patients enrolled in the present study showed reactivation of hepatitis within 12 months after discontinuing lamivudine administration, with 15 (75%) showing reactivation within 6 months. The peak serum ALT levels in the 20 reactivation patients ranged from 103 to 1019 IU/L, with a median of 308 IU/L. After lamivudine cessation, the maximum serum HBV DNA was significantly higher ($P < 0.001$) in the reactivation patients (median 7.8, 25–75% range 7.4–8.1 log copy/mL) than in the non-reactivation patients (median 4.8, 25–75% range 4.1–5.9 log copy/mL).

Table 1 shows a comparison of the clinical backgrounds at the onset and completion of lamivudine administration between the two groups of patients. Although backgrounds were similar between the two groups just prior to lamivudine administration, HBcrAg levels were significantly higher in the reactivation patients after treatment. Both HBV DNA levels and positive rates of HBe antigen were similarly low between the two groups. The duration of undetectable HBV DNA before stopping lamivudine administration was also similar ($P > 0.2$) between the two groups (reactivation patients, median 11 months, 25–75% range 8–13 months vs. non-reactivation patients, median 6 months, 25–75% range 5–13 months).

In 23 patients who were negative for HBe antigen after treatment, HBcrAg levels were significantly higher ($P = 0.011$) in the reactivation patients ($n = 12$, median 4.8 log U/mL, 25–75% range 4.0–5.0 log U/mL) than in non-reactivation patients ($n = 11$, median 3.0 log U/mL, 25–75% range 2.5–4.4 log U/mL). In contrast, levels were similar ($P > 0.2$) between the two groups in 11 patients who were positive for HBe antigen after treatment (reactivation patients $n = 8$, median 5.9 log U/mL, 25–75% range 5.1–6.1 log U/mL vs. non-reactivation patients $n = 3$, median 5.6 log U/mL, 25–75% range 2.5–8.0 log U/mL).

The ability of HBcrAg concentration to predict non-recurrence of hepatitis was analyzed using a ROC curve (Fig. 1), and the area under the curve was as wide as 0.764. The point at which specificity was 0.8 and sensi-

Table 1 Comparison of clinical characteristics at the onset and cessation of lamivudine administration between patients with and without reactivation of hepatitis

Characteristics	Reactivation of hepatitis		*P*-value†
	Positive ($n = 20$)	Negative ($n = 14$)	
Demographics			
Age (years)	44 (38–51)	50 (35–59)	NS
Sex (male/female)	13/7	7/7	NS
HBV genotype (B/C)	0/16	2/9	NS
At onset of lamivudine administration			
ALT (IU/mL)	103 (57–234)	211 (76–515)	NS
HBeAg (positive)	12 (60%)	4 (29%)	NS
HBV DNA (log copy/mL)	7.1 (6.1–8.1)	6.0 (5.3–7.4)	NS
HBcrAg (log unit/mL)	6.2 (5.6–7.7)	6.4 (5.0–6.6)	NS
At cessation of lamivudine administration			
Duration of lamivudine (months)	12.7 (10.4–16.3)	10.3 (6.4–17)	NS
ALT (IU/mL)	30 (15–36)	21 (15–24)	NS
HBeAg (positive)	8 (40%)	3 (21%)	NS
HBV DNA (log copy/mL)	<3.7 (<3.7–<3.7)	<3.7 (<3.7–<3.7)	NS
HBcrAg (log unit/mL)	4.9 (4.7–5.9)	3.2 (<3.0–4.5)	0.009

†Analysis of continuous variables performed using Mann–Whitney *U*-test; analysis of dichotomous variables performed using Fisher's exact test. Values shown as median (25–75% range) or *n* (%).
ALT, alanine aminotransferase; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

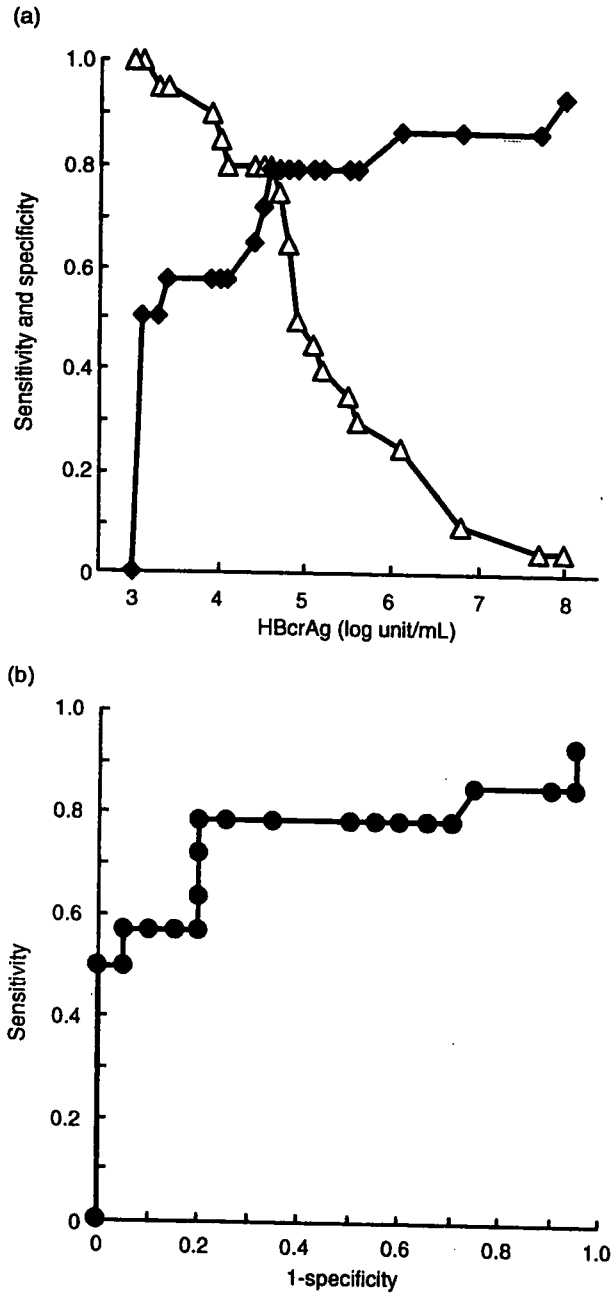

Figure 1 Receiver-operator characteristic (ROC) analysis of hepatitis B core-related antigen (HBcrAg) concentration for predicting patients without risk of reactivation of hepatitis within 12 months after halting lamivudine administration. (a) Sensitivity (■) and specificity (△) curves according to concentration of HBcrAg. (b) The ROC curve with the area under curve of 0.764.

tivity approximately 0.8 was deemed best for halting treatment without the risk of hepatitis recurrence. This point corresponds to an HBcrAg concentration of 4.1–4.6 log unit/mL.

DISCUSSION

THE REACTIVATION OF hepatitis following lamivudine administration was defined in the present study as an elevation of serum ALT level to more than 80 IU/L because we sought to find a more reliable indicator for safer discontinuation of lamivudine administration. Under these conditions, the majority (20/34) of patients showed reactivation of hepatitis within 12 months, as has been previously reported.[5,6] HBV DNA levels at the time of discontinuing lamivudine were similarly low between the two groups of patients, which is understandable as an undetectable reading typically indicates HBV remission following lamivudine therapy. However, HBcrAg levels were significantly higher in reactivation patients, implying that HBcrAg level is a better marker than HBV DNA level for predicting non-reactivation of hepatitis after discontinuing lamivudine administration especially in patients without HBe antigen.

In this study, ROC curve analyses showed a wide area under the curve of 0.764 in predicting the non-reactivation of HBV with HBcrAg level. If the corresponding cutoff is set at 4.5 logU/mL, then both specificity and sensitivity are as high as approximately 0.8. To obtain a higher specificity of 0.9, the cutoff value of HBcrAg concentration should be set at 4.0 log unit/mL. In this case, the sensitivity would still be nearly 0.6. The cutoff value of HBcrAg for predicting the non-relapse of hepatitis in our study is a little higher than that reported by Shinkai *et al.* (3.4 logU/mL).[14] Because numbers of patients analyzed were small in both studies, further studies are required to confirm the most appropriate cutoff value. It is noteworthy that this cutoff value may also differ among genotypes, which have been reported to be correlated with outcome of chronic HBV infection.[15] However, as over 90% of the patients had genotype C in this study, reactivation could not be analyzed in relation to HBV genotypes.

The HBV is an enveloped DNA virus containing a relaxed circular DNA genome which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells and serves as transcriptional template for the production of viral RNA.[11,16,17] Reverse transcription of pregenomic RNA and second-strand DNA synthesis then occur in the cytoplasm within viral

capsids formed by the HBV core protein. Because lamivudine inhibits reverse transcription of pregenomic RNA, it directly suppresses production of HBV virions, and serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. However, the production of viral proteins is not suppressed by lamivudine as this process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which also serves as a template for mRNAs, decreases quite slowly after commencement of administration of nucleoside analogs.[18,19] Thus, it is possible that serum HBcrAg levels reflect the cccDNA level in hepatocytes more accurately than serum HBV DNA. High levels of cccDNA are considered to be associated with hepatitis reactivation because they preceed reactivation of viral replication and consequent elevation of HBV DNA level in serum.

Lamivudine has already been eliminated from first line therapy in naïve chronic hepatitis B patients due to a higher incidence of developing resistant mutations than new antiviral agents, such as adefovir dipivoxil and entecavir.[20] However, the distinct characteristic of the HBcrAg assay under lamivudine therapy that is different from other HBV DNA assays is that lamivudine suppresses production of HBV virions by inhibiting reverse transcription of pregenomic RNA, but does not suppress the production of viral proteins, in which reverse transcription is unnecessary. Thus, it is possible that the HBcrAg assay may also useful for patients undergoing entecavir or adefovir dipivoxil administration because the main mechanism of suppressing HBV replication is similar between lamivudine and other antiviral agents. As a considerable number of patients who started lamivudine administration in the past are still taking this treatment now, the present study may be valuable for such patients when they consider changing therapies in the future. Additionally, further studies are required to determine whether the HBcrAg assay is indeed applicable to antiviral agents other than lamivudine.

In conclusion, significant markers that can predict reactivation of hepatitis after discontinuing lamivudine administration are clinically valuable because the reactivation of hepatitis is a fundamental problem in lamivudine therapy. Our results suggest that patients with an HBcrAg level of less than 4.5 log unit/mL may stop lamivudine administration with a lower risk of reactivation. The present study is a preliminary one because the patients enrolled were selected retrospectively without standardized criteria for stopping lamivudine and the number of patients enrolled was not large; however, the results may be valuable for patients with hepatitis B undergoing lamivudine therapy as such a diagnostic marker has rarely been reported. Further studies are required to establish the clinical significance of the HBcrAg assay in the treatment of hepatitis B.

ACKNOWLEDGMENT

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REFERENCES

1 Dienstag JL, Goldin RD, Heathcote EJ *et al.* Histological outcome during long-term lamivudine therapy. *Gastroenterology* 2003; 124: 105–17.
2 Lai CL, Chien RN, Leung NW *et al.* A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; 339: 61–8.
3 Liaw YF, Sung JJ, Chow WC *et al.* Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; 351: 1521–31.
4 Matsumoto A, Tanaka E, Rokuhara A *et al.* Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B. A multicenter retrospective study of 2795 patients. *Hepatol Res* 2005; 32: 173–84.
5 Liaw YF, Chien RN, Yeh CT, Tsai SL, Chu CM. Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. *Hepatology* 1999; 30: 567–72.
6 Lok AS, Lai CL, Leung N *et al.* Long-term safety of lamivudine treatment in patients with chronic hepatitis B. *Gastroenterology* 2003; 125: 1714–22.
7 Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; 40: 439–45.
8 Rokuhara A, Tanaka E, Matsumoto A *et al.* Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. *J Viral Hepat* 2003; 10: 324–30.
9 Bruss V, Gerlich WH. Formation of transmembraneous hepatitis B e-antigen by cotranslational *in vitro* processing of the viral precore protein. *Virology* 1988; 163: 268–75.
10 Garcia PD, Ou JH, Rutter WJ, Walter P. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. *J Cell Biol* 1988; 106: 1093–104.
11 Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; 337: 1733–45.
12 Tanaka E, Matsumoto A, Suzuki F *et al.* Measurement of hepatitis B virus core-related antigen is valuable for iden-

tifying patients who are at low risk of lamivudine resistance. *Liver Int* 2006; 26: 90–6.
13 Mizokami M, Nakano T, Orito E *et al.* Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 1999; 450: 66–71.
14 Shinkai N, Tanaka Y, Orito E *et al.* Measurement of hepatitis B virus core-related antigen as predicting factor for relapse after cessation of lamivudine therapy for chronic hepatitis B virus infection. *Hepatol Res* 2006; 36: 272–6.
15 Fung SK, Lok AS. Hepatitis B virus genotypes: do they play a role in the outcome of HBV infection? *Hepatology* 2004; 40: 790–2.
16 Mason WS, Halpern MS, England JM *et al.* Experimental transmission of duck hepatitis B virus. *Virology* 1983; 131: 375–84.
17 Tuttleman JS, Pourcel C, Summers J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell* 1986; 47: 451–60.
18 Moraleda G, Saputelli J, Aldrich CE, Averett D, Condreay L, Mason WS. Lack of effect of antiviral therapy in nondividing hepatocyte cultures on the closed circular DNA of woodchuck hepatitis virus. *J Virol* 1997; 71: 9392–9.
19 Werle-Lapostolle B, Bowden S, Locarnini S *et al.* Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 2004; 126: 1750–8.
20 Lok ASF, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; 45: 507–39.

Hepatology Research 2007; 37: 425–432 doi: 10.1111/j.1872-034X.2007.00050.x

Original Article

Pathological analysis of oxyphilic granular hepatocytes and hepatocellular mitochondria in chronic hepatitis C

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Aim: Oxyphilic granular hepatocyte (OGH) results from hepatocellular changes associated with chronic hepatitis. The histopathological significance of OGH has not been clarified.

Methods: The subjects consisted of two groups of patients with hepatitis C: one group of patients who had undergone liver biopsy 3.8 times on average, and were followed for 8 years on average, and one group of hepatocellular carcinoma (HCC) patients who had undergone hepatectomy. The following items were examined: frequency of OGH, relationship between OGH and the degree of fibrosis and inflammation; amount of mitochondria in resected tissues; activity of mitochondrial enzymes; relationship between the development of HCC and OGH; and relationship between the duration of infection and OGH in the post-transfusion patients.

Results: The incidence of OGH was 35.3% in liver biopsy patients and 46.9% in resected patients. A higher stage of fibrosis was associated with a higher frequency of OGH. Not only OGH but also hepatocyte mitochondria in the peripheral zone increased with the progression of fibrosis. Hepatocytes with or without increased mitochondria were randomly distributed. The mitochondrial enzyme activity was increased in hepatocytes with increased mitochondria. In the post-transfusion patients, a longer duration of infection and a higher stage of fibrosis were associated with a higher frequency of OGH. A high percentage of patients with OGH developed HCC.

Conclusion: Mitochondrial changes are important histological findings related to the progression of liver lesions and the possible development of HCC.

Key words: cytochrome c oxydase, hepatitis C, mitochondria, oxyphilic granular hepatocyte, succinate dehydrogenase

INTRODUCTION

VARIOUS HEPATOCELLULAR CHANGES are observed in viral hepatitis, including oxyphilic granular hepatocyte (OGH). Characteristic acidophilic changes in the cell have been reported to be due to mitochondrial hyperplasia (Fig. 1). OGH was first discovered in hepatitis B by Lefkowitch *et al.*[1] They have extensively examined OGH and clarified the pathological profile of OGH in hepatitis B, although the real pathogenesis remains unknown.[1–3] OGH has also been found in other liver diseases such as primary biliary cirrhosis and alcoholic hepatitis. OGH in hepatitis C has not been analyzed in detail. There is one report that documented OGH in patients with various diseases including hepatitis C. Muller-Hocker found OGH in 20 of 47 cirrhosis livers of various etiology, but the frequency of OGH in patients with hepatitis C was not reported.[4] In this study, we examined the pathological features, pathogenesis and significance of OGH in hepatitis C.

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METHODS

Subjects

THE SUBJECTS WERE two groups of patients with hepatitis C: one group comprised patients who had undergone liver biopsy and the other group comprised patients who had undergone liver resection.

Biopsy group

Fifty-one HCV antibody-positive patients with hepatitis C were studied. These patients had been analyzed and reported previously by one of the authors.[5] The patients had been diagnosed with chronic hepatitis C, treated, and followed between 1967 and 1994 in the National

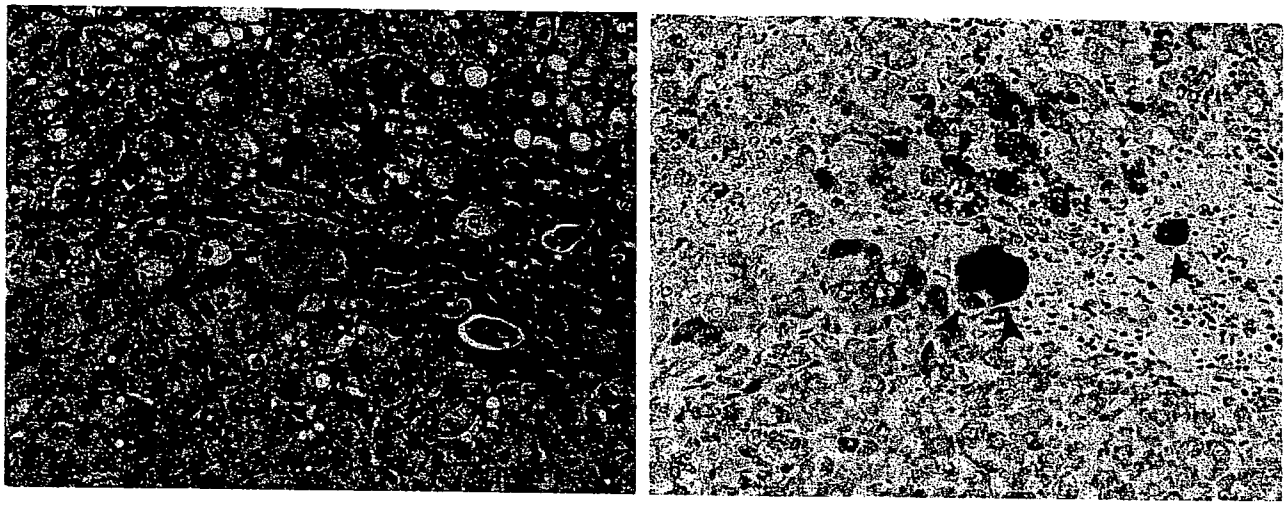

Figure 1 (a) A typical oxyphilic granular hepatocyte (arrowhead) is filled with acidophilic granules (hematoxylin and eosin). (b) A section serial to that in (a) was immunohistochemically stained with antimitochondrial antibody. Mitochondrial hyperplasia was observed in the oxyphilic granular hepatocyte. The arrowhead indicates cells corresponding to those shown in (a). (Original magnification ×400.)

Hospital Organization Nagasaki Medical Center. They usually underwent the first liver biopsy within 6 months of onset, and two or more liver biopsies during follow-up, with a mean of 3.8 biopsies and a follow-up period of 1–21 years (mean 8 years).

Resected group

Sixty-four patients with resected hepatitis C-related hepatocellular carcinoma (HCC) who had undergone hepatectomy from 2001 to 2002 were studied. Eighteen resected patients who had metastatic colorectal cancer during the same period served as controls. Frozen liver tissues from 37 patients with hepatitis C-related HCC and four patients with metastatic colorectal cancer had been stored, and were used as frozen sections for histochemical staining.

Histological examination

Liver biopsy and resected tissues were fixed in 10% formalin and embedded in paraffin. Sections 4-μm thick were stained with hematoxylin and eosin, and examined for OGH and their distribution within the liver lobule. In addition, the degree of liver tissue fibrosis and inflammation was evaluated according to the Ludwig classification of chronic hepatitis.[6]

To examine the relationship between the duration of infection and the appearance of OGH, biopsy patients with post-transfusion hepatitis C were analyzed for the time interval between transfusion and the appearance of OGH, and the degree of fibrosis and inflammation at the time of the appearance of OGH. The frequency of OGH in the liver biopsy patients who developed HCC during follow-up was also determined.

Amount of mitochondria in hepatocytes

To objectively evaluate the amount of mitochondria in resected tissues, immunohistochemical staining with antimitochondrial antibody (Bio Genex, San Ramon, CA, USA) was performed. The amount of mitochondria in the entire liver of each patient was expressed as follows. The amount of mitochondria in each hepatocyte was classified as large, equal, or small, in comparison with that in a control hepatocyte. Five areas per patient were randomly selected from the peripheral zone. The amount of mitochondria in about 500 hepatocytes in each area was classified.

To semiquantify the amount of mitochondria in liver tissue, the percentages of cells with a large, equal, and small amount of mitochondria to the total number of cells observed were calculated to score the amount of mitochondria according to the following formula: Mitochondrial score (MS) = large amount (%) × 3 + equal amount (%) × 2 + small amount (%) × 1.

Hepatocyte mitochondrial function

To evaluate hepatocyte mitochondrial function, frozen sections of resected liver tissues were histochemically stained for the electron transport enzymes succinate dehydrogenase (SDH) and cytochrome c oxydase (COX).

For SDH, the following substrate solution was used: 100 mM disodium succinate, 25 mM phosphate-

buffered saline (PBS; pH 7.4), 1 mM NaN_3, 0.2 mM PMS, and 1.5 mM NitroBT. Unfixed 5-mm thick sections were incubated in the substrate solution at 37°C for 5 min, briefly rinsed in PBS and then water, and sealed with glycerin-gelatin. The substrate disodium succinate was omitted in the processing of controls.[7–9]

For COX, the following substrate solution was used: 50 mM PBS (pH 7.2), 0.15% cytochrome c, and 2.5 mM DAB. Unfixed 10-mm thick sections were incubated in the substrate solution at 37°C for 10 min, rinsed well in PBS, dehydrated in a series of ethanol, cleared in xylene, and mounted with a synthetic mount. The substrate cytochrome c was omitted in the processing of controls.[7,8,10]

Changes in hepatocytes

Resected sections (five OGH positive, five OGH negative, and five control) were immunohistochemically stained with anti-hexokinase (HXK)-II antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for the glycolysis enzyme,[11,12] and with anti-Ki-67 antibody (Novo Castra, Newcastle Upon Tyne, UK) for the cell growth cycle. The expression of Ki-67 was evaluated in 10 high-power magnification areas in the peripheral zone and the central zone to calculate the labeling index (LI).

Statistical analysis

The frequency and distribution of OGH and the relationship between OGH and the degree of fibrosis and inflammation were assessed. The Mann–Whitney *U*-test and chi-squared test were employed. $P < 0.05$ was considered statistically significant.

RESULTS

Biopsy group

AT THE TIME of the first liver biopsy, the frequencies of OGH in patients with stage 3 and 4 fibrosis were 18.2% and 28.6%, respectively, but OGH did not appear in patients with stage 1 or 2 fibrosis. OGH appeared in 16.7% of patients with grade 4 portal tract inflammation and 7.7% of patients with grade 3 portal tract inflammation. OGH first appeared at the age of 48–69 years, with a mean of 56.9 years.

Patients in whom OGH appeared at least once were classified as OGH-positive. OGH appeared in 18 (35.3%) of the 51 liver biopsy patients. At the first appearance of OGH, stage 4 fibrosis was observed in 72.2% of the biopsy patients. A more advanced stage of fibrosis tended to be associated with a higher frequency of OGH. At the first appearance of OGH, inflammation tended to be severe in the portal tract, but mild in the liver lobule. Grade 4 portal inflammation was noted in 94.4% of the 18 OGH-positive patients (Fig. 2).

Of the 51 liver biopsy patients, 21 (41.2%) had received blood transfusions, and OGH appeared in six (28.6%) of them. The time interval between blood

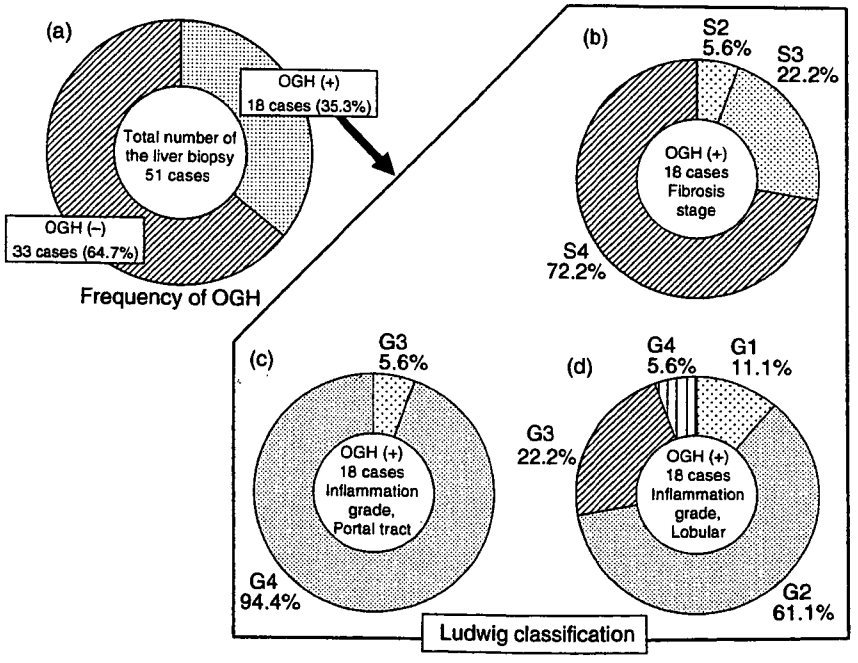

Figure 2 The fibrosis stage and inflammation grade at the first appearance of oxyphilic granular hepatocyte (OGH) on liver biopsy in relation to the frequency of OGH. (a) OGH appeared in 18 (35.3%) of the 51 liver biopsy patients. (b) OGH was observed in 94.4% of patients with stage 3 or 4 fibrosis. (c) OGH appeared in all patients with grade 3 or 4 portal tract inflammation. (d) No relationship was found between the grade of lobular inflammation and the appearance of OGH.

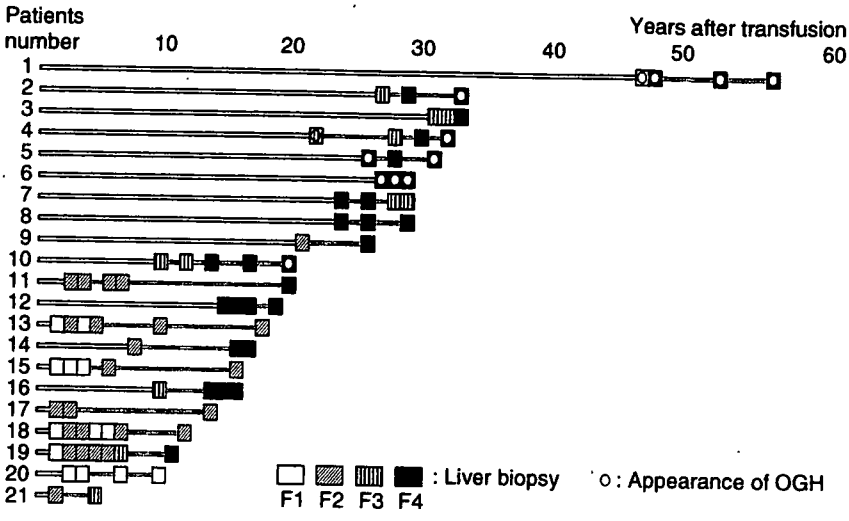

Figure 3 Post-transfusion course of patients. The mean time interval between blood transfusion and the appearance of oxyphilic granular hepatocyte (OGH) was 28 years and 4 months (range 20–40 years). OGH tended to appear in patients with severe liver fibrosis at more than 20 years after blood transfusion. In contrast, OGH was not observed in patients with severe liver fibrosis at less than 20 years after blood transfusion.

transfusion and the appearance of OGH ranged from 20 to 40 years, with a mean of 28 years and 4 months (Fig. 3).

Hepatocellular carcinoma developed in 17 (33.3%) of the 51 liver biopsy patients, and OGH was observed in eight (47.1%) of them. HCC was not observed in 34 patients, in 10 (29.4%) of whom OGH appeared (Fig. 4). At the last liver biopsy, 21 patients with stage 3–4 fibrosis and two patients with stage 1–2 fibrosis had developed HCC. Therefore, 20 patients with stage 3–4 fibrosis and eight patients with stage 1–2 fibrosis did not develop HCC. No significant difference was noted in stage of fibrosis between the patients who did or did not develop HCC ($P = 0.0914$)

Resected group

Oxyphilic granular hepatocyte appeared in 30 (46.9%) of the 64 resected patients (Fig. 5), but in none of the control patients. Inflammatory cell infiltration was prominent, especially in periportal areas with piecemeal necrosis (Fig. 1). The frequency of OGH tended to become higher with the progression of fibrosis (Fig. 5), and was significantly higher in the group with severe fibrosis than in the group with mild fibrosis (Table 1). A higher grade of portal tract inflammation tended to be associated with a higher frequency of OGH: the frequency was significantly higher in the group with severe portal tract inflammation than in the group with mild portal tract inflammation (Table 1), but did not significantly differ with respect to the degree of hepatic lobular inflammation (Table 1). Thus, the histological findings in the resected patients were similar to those of the biopsy patients.

Amount of mitochondria in hepatocytes

The amount of hepatocyte mitochondria was evaluated in the resected patients. Immunohistochemical staining with antimitochondrial antibody was strongly positive in OGH (Fig. 1). Mitochondria were also increased in hepatocytes other than OGH. However, in patients with stage 3–4 fibrosis, the amount of mitochondria was decreased markedly in some hepatocytes in the central

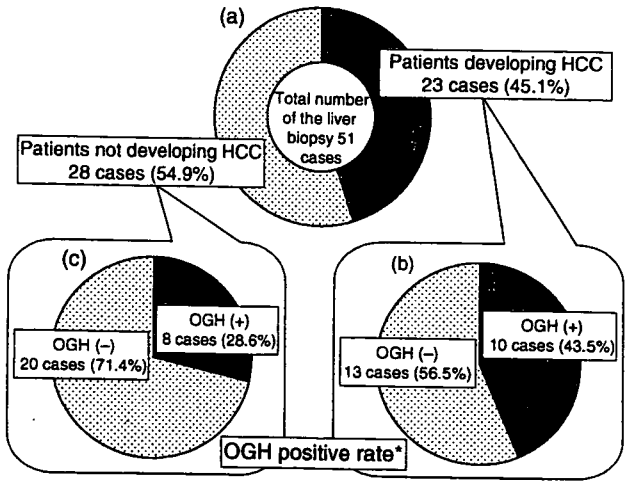

Figure 4 The frequency of oxyphilic granular hepatocyte (OGH) in patients who developed hepatocellular carcinoma (HCC). (a) HCC developed in 23 (45.1%) of the 51 liver biopsy patients. (b) OGH appeared in 10 (43.5%) of the 17 patients who developed HCC. (c) OGH appeared in 8 (28.6%) of the 34 patients who did not develop HCC. No significant difference was noted in the frequency of OGH between the patients who did or did not develop HCC (*$P = 0.4156$, chi-squared test).

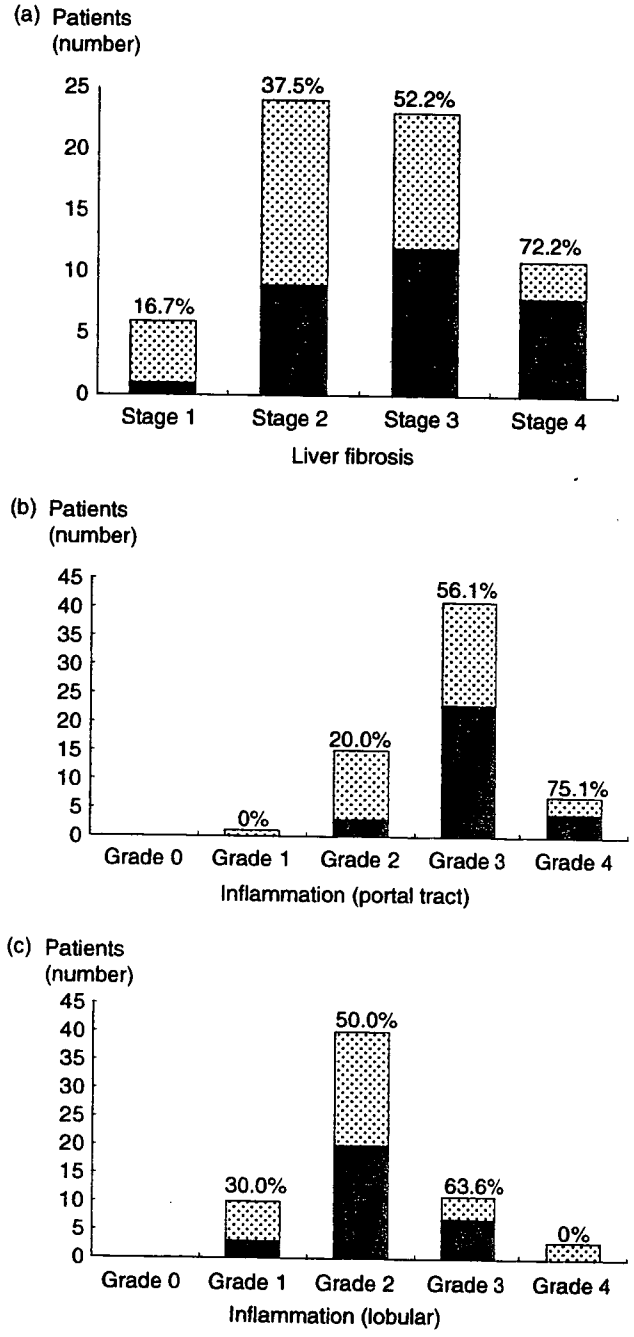

Figure 5 The fibrosis stage and inflammation grade in resected patients in relation to the frequency of oxyphilic granular hepatocyte (OGH). (a) A higher stage of fibrosis was associated with a higher frequency of OGH. (b) A higher grade of portal tract inflammation was associated with a higher frequency of OGH. (c) No relationship was found between the grade of lobular inflammation and the appearance of OGH. (⊡), OGH (–); (■), OGH (+).

Table 1 Fibrosis stage and inflammation grade (Ludwig classification) in relation to the frequency of OGH in the resected patients

Parameter	OGH	No OGH	*P*-value†
Stage			
1–2	10	20	0.041
3–4	20	14	
Grade (portal)			
0–2	3	13	0.009
3–4	27	21	
Grade (lobular)			
0–2	23	27	0.167
3–4	7	7	

†Chi-squared test, frequency of OGH in stage 1–2 *vs* 3–4 or grade 0–2 *vs* 3–4.
OGH, oxyphilic granular hepatocyte.

zone, but only slightly in the peripheral zone (Fig. 6). The mean mitochondrial score for the peripheral zone was 134.47 ± 27.24 (range 100–196) in the group with mild fibrosis, 156.82 ± 30.14 (range 107–227) in the group with severe fibrosis, and 128.89 ± 24.03 (range 100–193) in the control group. Thus, the score for the peripheral zone was significantly higher in the severe than in the mild fibrosis group. The amount of mitochondria in the peripheral zone tended to increase with the progression of fibrosis (Fig. 7).

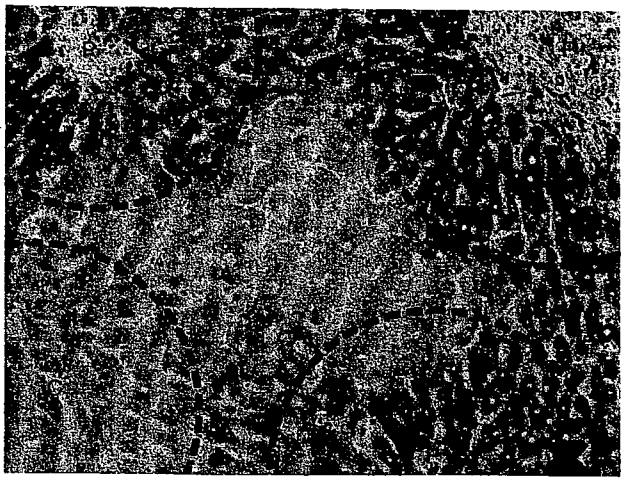

Figure 6 Immunohistochemical staining with antimitochondrial antibody. Hepatocytes with increased or decreased mitochondria were distributed in an irregular, map-like manner. The amount of mitochondria in hepatocytes was increased or maintained in the peripheral zone (P), but was markedly decreased in the central zone (C) in some patients. (Original magnification ×40.)

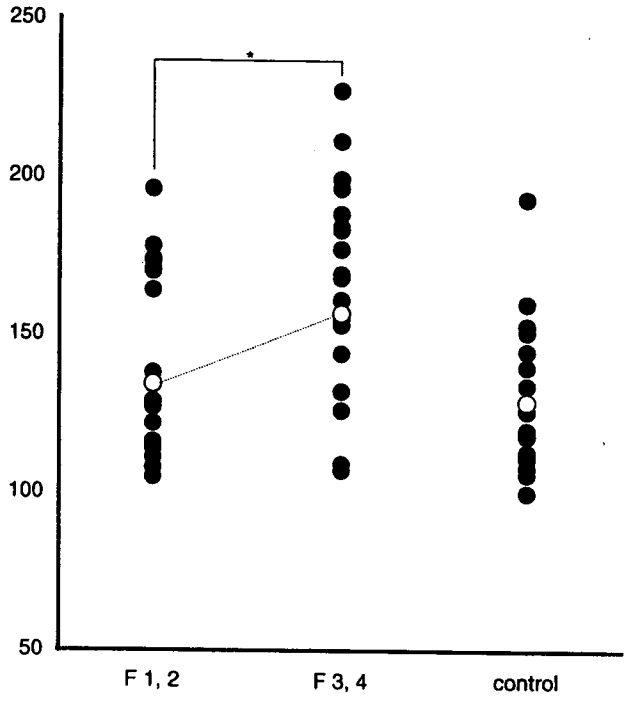

Figure 7 The relationship between the mitochondrial score and liver fibrosis in the peripheral zone. The mean mitochondrial scores for peripheral zone in the group with mild fibrosis, the group with severe fibrosis, and the control group were 134.47 ± 27.24 (100–196), 156.82 ± 30.14 (107–227), and 128.89 ± 24.03 (100–193), respectively. Thus, the mitochondrial score for peripheral zone was significantly higher in the group with severe fibrosis than in the group with mild fibrosis (*$P = 0.011$, Mann–Whitney *U*-test).

Hepatocyte mitochondrial function

Immunohistochemical staining with antimitochondrial antibody showed increased SDH and COX activity in the area with an increased amount of mitochondria (Fig. 8).

Changes in hepatocytes

There was increased expression of HXK-II in hepatocytes with increased mitochondria (Fig. 9), but not in control cases.

The mean Ki-67 labeling indices for the peripheral and central zones were 0.56 and 0.47, respectively, showing no significant differences among zones.

DISCUSSION

LEFKOWITCH *ET AL.* examined OGH in hepatitis B.[1] The frequencies of OGH in liver biopsy specimens in their study and ours were 41.7% and 35.5%, respectively, which appear to be roughly similar. They reported that OGH tended to appear in hepatitis B patients with severe fibrosis and inflammatory cell infiltration: among the liver biopsy specimens from their hepatitis B patients, the frequency of OGH was as high as 54.5%, whereas no OGH was observed in patients with nonactive cirrhosis. We determined the stage of fibrosis and grade of inflammation according to the Ludwig classification of chronic hepatitis.[6] In chronic hepatitis C, the frequency of OGH tended to be high in patients with severe fibrosis and portal tract inflammation (stage 3–4 or portal grade 3–4), and no OGH appeared in patients with mild fibrosis and portal tract inflammation (stage 1–2 or portal grade 1–2). Although it is difficult to accurately compare their results with ours, because of the difference in the method of classification of chronic hepatitis, we consider that the histopathological features of the background liver in which OGH appears are similar in hepatitis B and C. A similar tendency was observed in the resected patients.

No studies have examined the relationship between the appearance of OGH and the duration of hepatitis B. The present study showed that the duration of infection

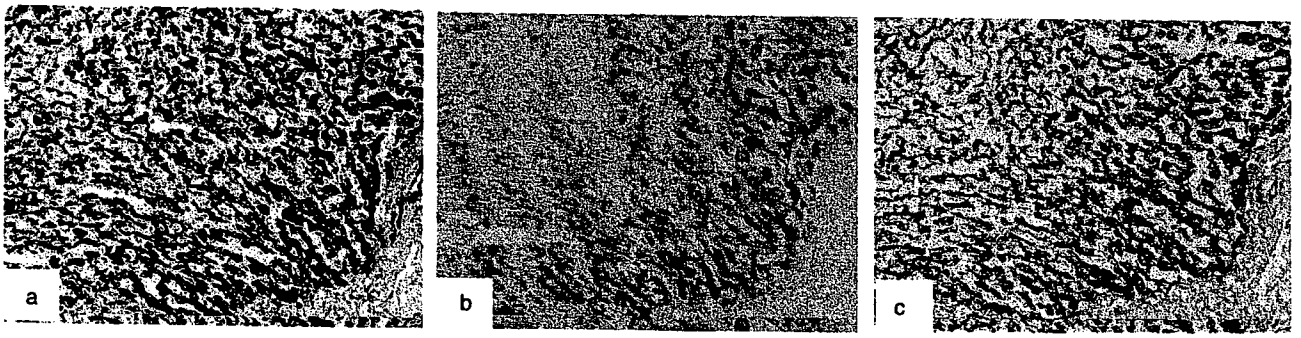

Figure 8 Serial frozen sections stained (a) with antimitochondrial antibody, (b) for succinate dehydrogenase (SDH), or (c) for cytochrome c oxidase (COX). SDH- and COX-positive cells were stained deep blue and brown, respectively. The expression of SDH and COX was increased in hepatocytes with increased mitochondria.

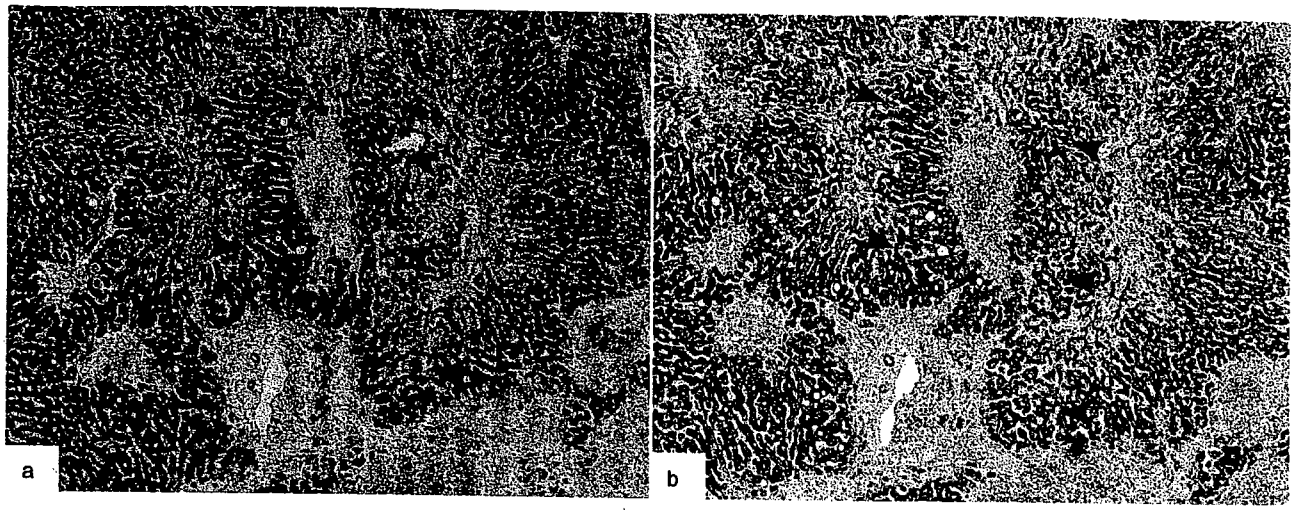

Figure 9 Immunohistochemical staining with (a) antimitochondrial antibody and (b) anti-hexokinase (HXK)-II antibody, showing HXK-II expression was increased in hepatocytes with increased mitochondria. (Original magnification ×40.)

in hepatitis C was closely related to the appearance of OGH: a longer duration of infection and a more advanced stage of fibrosis tended to be associated with a higher frequency of OGH. In patients with a long duration of infection, OGH appeared in the absence of cirrhosis. In contrast, in patients with an infection duration of less than 20 years, OGH did not appear in the presence of severe fibrosis. These findings suggest that the duration of infection is a factor in the development of OGH, which is related to long-term chronic infection. With little supporting evidence, we can only speculate that the persistence of chronic infection, hepatitis C virus infection of mitochondria, and oxidant stress for more than 20 years may alter mitochondria.

Immunohistochemical staining with antimitochondrial antibody allowed us to more objectively evaluate mitochondria in hepatocytes, and showed that mitochondria were increased not only in OGH, but also in other hepatocytes. Conversely, hepatocytes with markedly reduced mitochondria were clearly identified. An increase, but not a decrease, in the amount of hepatocyte mitochondria in chronic liver disease has been reported in several reports. Moreover, hepatocytes with increased or decreased mitochondria were distributed in an irregular, map-like manner. However, hepatocytes with increased mitochondria tended to be more frequently found in the peripheral zone, and those with decreased mitochondria in the central zone. It has been reported that chronic viral hepatitis is histologically characterized by the irregular distribution of liver cell necrosis and regeneration.[13–15] The irregular distribution of hepatocytes with increased or decreased mitochondria in this study may be related to the previously reported irregular distribution of lesions of chronic viral hepatitis.

Among the hepatitis C patients studied, OGH appeared in 47.1% of the liver biopsy patients and 46.9% of the resected patients with HCC. Interestingly, the frequency of OGH was higher in the patients with than those without HCC among the liver biopsy patients, although no significant difference was noted. It has been reported that HCC develops commonly in cirrhotic patients, but rarely in non-cirrhotic patients.[16,17] HCC has been reported to develop in as high as 3–10% of hepatitis C-related cirrhotic patients yearly.[18,19] A more advanced stage of fibrosis has been reported to be associated with a higher incidence of complication by HCC. Considering that OGH frequently developed in HCC patients in association with a long duration of infection, the stage of fibrosis and the appearance of OGH in biopsy tissue may serve as indicators predicting the development of HCC.

Hypotheses about the pathogenesis of OGH have been proposed.[20–25] As immunohistochemical staining showed no differences among zones in the expression of Ki-67, which reflects the cell growth cycle, it is difficult to consider that OGH is regenerated hepatocytes. Tanji *et al.*[26] performed histochemical studies of OGH in chronic hepatitis, and found deficiencies of mtDNA and mtDNA-encoded respiratory chain enzymes in OGH. We evaluated the function of the increased mitochondria, and obtained results different from theirs. Histochemical staining and immunohistochemical staining with antimitochondrial antibody showed increased activity of electron transport enzymes in the

area of increased mitochondria, indicating that there was no functional impairment of the hyperplastic mitochondria. In addition, the increased staining for HXK-II suggests an increased glycolytic pathway. The increase in the glycolytic pathway and the increased activity of electron transport enzymes indicate that energy production was markedly enhanced. In this study, we did not perform DNA analysis, a factor to be studied in the future. The presence of areas of increased and markedly decreased mitochondria suggests that the former area represents compensatory mitochondrial hyperplasia for the latter area. OGH may be an extreme form of compensatory hyperplastic change.

REFERENCES

1 Lefkowitch JH, Arborgh BA, Scheuer PJ. Oxyphilic granular hepatocytes. Mitochondrion-rich liver cells in hepatic disease. *Am J Clin Pathol* 1980; 74: 432–41.
2 Gerber MA, Thung SN. Hepatic oncocytes. Incidence, staining characteristics, and ultrastructural features. *Am J Clin Pathol* 1981; 75: 498–503.
3 Altmann HW. [Oncocytic hepatocytes.] *Pathologe* 1990; 11: 137–42 (in German).
4 Muller-Hocker J. Defects of the respiratory chain in hepatic oncocytes. *Virchows Arch* 1998; 432: 349–56.
5 Kage M, Shimamatu K, Nakashima E *et al.* Long-term evolution of fibrosis from chronic hepatitis to cirrhosis in patients with hepatitis C: morphometric analysis of repeated biopsies. *Hepatology* 1997; 25: 1028–31.
6 Ludwig J. The nomenclature of chronic active hepatitis: an obituary. *Gastroenterology* 1993; 105: 274–8.
7 Stoward PJ, Pearse AGE. Histochemical methods for dehydrogenases. In: Stoward PJ, Pearse AGE, eds. *Histochemistry. Vol. 3 Enzyme Histochemistry*, 4th edn. London: Churchill Livingstone, 1991; 537–72.
8 Woltsche M, Zatloukal K, Denk H. Enzyme-histochemical studies of griseofulvin-intoxicated mouse livers. *Liver* 1991; 11: 231–40.
9 Chieco P, Moslen MT, Reynolds ES. Histochemical evidence that plasma and mitochondrial membranes are primary foci of hepatocellular injury caused by 1,1-dichloroethylene. *Lab Invest* 1982; 46: 413–21.
10 Kang YH, McKenna T, Watson LP *et al.* Cytochemical changes in hepatocytes of rats with endotoxemia or sepsis: localization of fibronectin, calcium, and enzymes. *J Histochem Cytochem* 1988; 36: 665–78.
11 Yasuda S, Arii S, Mori A *et al.* Hexokinase II and VEGF expression in liver tumors: correlation with hypoxia-inducible factor 1 alpha and its significance HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. *J Hepatol* 2004; 40: 117–23.
12 Deeb SS, Malkki M, Laakso M. Human hexokinase II: sequence and homology to other hexokinases. *Biochem Biophys Res Commun* 1993; 197: 68–74.
13 Shibata M, Morizane T, Uchida T *et al.* Irregular regeneration of hepatocytes and risk of hepatocellular carcinoma in chronic hepatitis and cirrhosis with hepatitis-C virus infection. *Lancet* 1998; 351: 1773–7.
14 Uchida T. Pathology of hepatitis C. *Intervirology* 1994; 37: 126–32.
15 Uchida T, Kronborg I, Peters RL. Acute viral hepatitis: morphologic and functional correlations in human livers. *Hum Pathol* 1984; 15: 267–77.
16 Colombo M. Natural history and pathogenesis of hepatitis C virus related hepatocellular carcinoma. *J Hepatol* 1999; 31 (Suppl 1): 25–30.
17 De Mitri MS, Poussin K, Baccarini P *et al.* HCV-associated liver cancer without cirrhosis. *Lancet* 1995; 345: 413–15.
18 Marcellin P. Hepatitis C: the clinical spectrum of the disease. *J Hepatol* 1999; 31 (Suppl 1): 9–16.
19 Alberti A, Chemello L, Benvegnu L. Natural history of hepatitis C. *J Hepatol* 1999; 31 (Suppl 1): 17–24.
20 Bassi M, Bernelli-Zazzera A. Ultrastructural cytoplasmic changes of liver cells after reversible and irreversible ischemia. *Exp Mol Pathol* 1964; 17: 332–50.
21 Confer DB, Stenger RJ. The evolution of lysosomes in hypoxic liver parenchyma as seen with the electron microscope. *Am J Pathol* 1964; 45: 533–46.
22 Oudea PR. Anoxic changes of liver cells. Electron microscopic study after injection of colloidal mercury. *Lab Invest* 1963; 12: 386–94.
23 Jordan SW. Electron microscopy of hepatic regeneration *Exp Mol Pathol* 1964; 86: 183–200.
24 Herdson PB, Garvin PJ, Jennings RB. Fine structural changes produced in rat liver by partial starvation. *Am J Pathol* 1964; 45: 157–81.
25 Lafontaine JG, Allard C. A light and electron microscope study of the morphological changes induced in rat liver cells by the azo dye 2-Me-Dab. *J Cell Biol* 1964; 22: 143–72.
26 Tanji K, Bhagat G, Vu TH *et al.* Mitochondrial DNA dysfunction in oncocytic hepatocytes. *Liver Int* 2003; 23: 397–403.

Toll-like Receptor Expression in Lupus Peripheral Blood Mononuclear Cells

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ABSTRACT. ***Objective.*** To investigate expression of members of the Toll-like receptor (TLR) family in peripheral blood mononuclear cells (PBMC) in patients with systemic lupus erythematosus (SLE).
Methods. We analyzed PBMC from 14 patients with SLE and 15 healthy subjects. The surface expressions of TLR2 and TLR4 and intracellular expression of TLR9 on PBMC were analyzed by flow cytometry.
Results. Although TLR4 expressions on CD14+ monocytes were not significantly different between healthy subjects and patients with SLE, TLR2 expressions on monocytes were reduced in patients with SLE compared to healthy subjects. Intracellular TLR9 expression levels of CD19+ B lymphocytes were significantly elevated in patients with SLE. However, the TLR9 expression levels of plasmacytoid dendritic cells were not significantly different between these patients and healthy subjects.
Conclusion. Our results show that human peripheral blood B cells express TLR9 and that its expression is increased in patients with SLE. This upregulated expression of TLR9 in B cells may be related to the abnormal B cell hyperactivity in patients with SLE. (First Release Jan 15 2007; J Rheumatol 2007;34:493–500)

Key Indexing Terms:
B LYMPHOCYTES
INTERFERON-α
SYSTEMIC LUPUS ERYTHEMATOSUS
TOLL-LIKE RECEPTOR

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the generation of autoantibodies against nuclear proteins and DNA[1]. These autoantibodies are thought to contribute to the pathogenesis of SLE, and the levels of anti-DNA antibodies correlate with the disease activity[2,3]. The deposition of antinuclear antibody and antigen immune complexes (IC) in tissue is thought to induce local activation of immune systems[4], which may perpetuate SLE. It has also been proposed that the type 1 interferon (IFN) system has a pivotal etiopathogenic role in SLE, since IFN-α correlates with disease activity of SLE[5,6]. Recent studies showed that the sera of patients with SLE selectively induce the production of IFN-α from the natural IFN-α-producing cells, which are identical to plasmacytoid dendritic cells (PDC)[7]. Further, DNA/anti-DNA antibody complexes in lupus sera can provide signals for IFN-α production through the Toll-like receptor-9 (TLR9), which responds to bacterial DNA sequences containing nonmethylated CpG motifs[8]. In humans, the TLR family consists of 10 members, each of which is involved in the recognition of pathogen-derived materials[9]. Recent data suggest that TLR also play an important role in autoimmunity[10]. It is important to determine whether circulatory mononuclear cells in patients with SLE express TLR that could be involved in PDC activation as well as IFN-α induction. We examined the expression of TLR in lupus circulating mononuclear cells using flow cytometry methods.

MATERIALS AND METHODS

Patients and controls. A total of 14 patients with SLE (12 women and 2 men, aged 36.1 ± 13.3 yrs) were enrolled in our study (Table 1), in addition to 15 healthy volunteers (12 women, 3 men, aged 37.7 ± 8.1 yrs) and 6 patients with rheumatoid arthritis (RA; 5 women, 1 man, aged 48.1 ± 11.4 yrs) as controls. Consecutive patients entering the rheumatology clinic who fulfilled the American College of Rheumatology 1982 revised classification criteria for SLE[11] were selected for this investigation. Among the 14 patients, 8 received prednisolone (PSL) as monotherapy (mean dosage 20.3 mg/day, range 5–60 mg/day). The remaining 5 patients were treated with both PSL (mean dosage 19.4 mg/day, range 7-50 mg/day) and cyclophosphamide (intermittent intravenous cyclophosphamide therapy), and one patient received PSL (5 mg/day) and azathioprine (50 mg/day). Disease activity was scored in all patients with SLE by SLE Disease Activity Index (SLEDAI)[12]. The study protocol was approved by the Ethics Committee of Nagasaki Medical Center.

Reagents for flow cytometry. Antibodies used for phenotype analysis were obtained from Beckman Coulter (Fullerton, CA, USA), and antibodies for TLR analysis were purchased from eBioscience (San Diego, CA, USA). Antibody for BDCA-2 was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Antibodies used in this study were as follows: FITC-labeled anti-human CD14, CD19, and BDCA-2; phycoerythrin (PE)-labeled anti-human TLR2, 4, and 9; and PC-5-labeled anti-human CD123.

Flow cytometry. Heparinized blood was collected and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation. For staining for TLR9, PBMC were stained for cell surface antigen and fixed

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Table 1. Clinical data of patients with SLE.

Patient No.	Age, yrs	Sex	SLEDAI	Anti-dsDNA, IU/ml	CH50, IU/ml	PSL, mg/day	Cyclophosphamide	Azathioprine
1	49	F	6	141	30	25		
2	27	F	4	26	30	11		
3	29	F	9	17	12	14		
4	19	F	5	5	36	5		+
5	27	F	8	21	29	10		
6	24	F	14	30	15	30		
7	57	F	4	5	38	7	+	
8	45	F	12	33	25	12.5	+	
9	25	F	16	14	26	60		
10	33	F	12	19	23	50	+	
11	38	F	2	17	38	5		
12	55	M	10	32	41	15	+	
13	24	M	18	17	29	15	+	
14	54	F	10	4	37	7.5		

SLEDAI: Systemic Lupus Erythematosus Disease Activity Index; anti-dsDNA: anti-double-stranded DNA antibody (normal 0.0–10.0 IU/ml); PSL: prednisolone.

and permeabilized using a commercial kit (eBioscience). Cells were then stained for intracellular TLR9 with a PE-labeled TLR9 monoclonal antibody (mAb; eBioscience). In brief, cells were stained for their surface markers using fluorescein isothiocyanate (FITC)-conjugated anti-human CD14, CD19, blood dendritic cell antigen (BDCA)-2, or PC-5-conjugated CD123 for 15 min. Cells were then washed with staining buffer, fixed, and permeabilized. After washing with staining buffer, cells were stained with PE-conjugated anti-human TLR9 (eBioscience). Fluorescence was measured on an Epics XL (Beckman-Coulter). The acquired data were analyzed with EXPO32 software (Beckman-Coulter).

Cell culture. PBMC were isolated by Ficoll-Hypaque (Amersham Pharmacia, Tokyo, Japan) density gradient centrifugation. T cells were removed from PBMC by negative selection using anti-CD2 magnetic beads (Dynal, Oslo, Norway). T-cell-depleted mononuclear cells (MNC; 1×10^5 per 200 μl well) were cultured in RPMI-1640 (Gibco, NY, NY, USA) supplemented with 10% fetal bovine serum (Gibco BRL Invitrogen, Tokyo) in 96-well tissue culture plates. CpG oligodeoxynucleotide (ODN) 2006 (5'-TCG TCG TTT TGT CGT TTT GTC GTT-3') complete phosphorothioate (CpG ODN2006; InvivoGen, San Diego, CA, USA) was added at Day 0. T-cell-depleted MNC were also cultured with 96-well plates coated with 1 μg/ml purified goat anti-human IgG, IgA, and IgG (Southern Biotechnology, Birmingham, AL, USA) to trigger the B cell receptor (BCR) as controls. Cell cultures were maintained for 4 days and culture supernatants were collected and stored at –20°C. The amounts of IgG in culture supernatants were determined by antibody sandwich-type ELISA (Bethyl Laboratories, Montgomery, AL, USA).

Statistical analysis. Comparisons between groups were done using the nonparametric Mann-Whitney U-test. All statistics were performed with Stat View 7.0 (SAS Institute, Cary, NC, USA).

RESULTS

TLR2 and TLR4 expressions on CD14+ cells in patients with SLE. We compared the baseline expression of TLR2 and TLR4 on CD14+ monocytes in patients with SLE and healthy subjects. As shown in a representative histogram of monocytes, surface expressions of TLR2 were lower in patients with SLE than in healthy subjects (Figure 1A). The geometric mean fluorescence intensity of TLR2 was significantly lower in patients with SLE than in healthy subjects (Figure 1B). However, there was no statistical difference in TLR4 expression between patients with SLE and healthy subjects (Figures 2A, 2B). Although we also examined the baseline expression of TLR2 and TLR4 on B cells and T cells in patients with

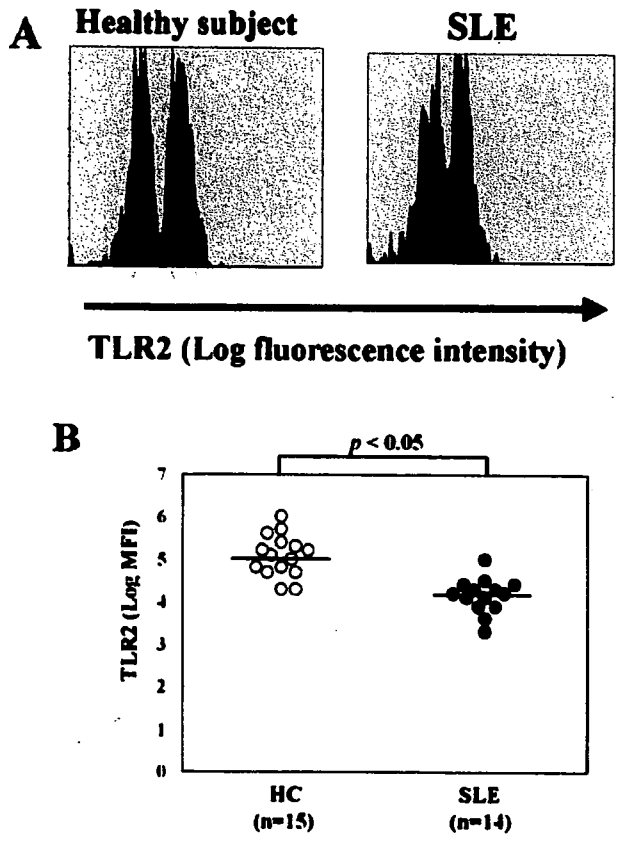


Figure 1. Diminished expression of TLR2 on CD14+ monocytes in patients with SLE. A. Histograms show baseline expression of surface TLR2 on monocytes in a representative patient with SLE and healthy subject. Darker areas indicate staining with isotype control mAb. B. Mean fluorescence intensity of surface TLR2 staining for CD14+ monocytes in patients with SLE (n = 14) and healthy subjects (HC; n = 15). Significance of differences between groups were analyzed by Mann-Whitney U-test.

SLE, we could not detect TLR2 and TLR4 expression on B or T cells in patients with SLE or in healthy subjects (data not shown).

Increased expression of TLR9 on B cells in patients with SLE. We also examined whether lupus B cells express TLR9 in patients with SLE. We identified B cells as CD19+ populations (Figures 3A, 3B). As shown in Figure 3C, CD19+ B cells express TLR9 constitutively. In a representative histogram of B cells, intracellular expression of TLR9 was higher in patients with SLE than in healthy subjects. The geometric mean fluorescence intensity of TLR9 in B cells is shown in Figure 3D. TLR9 expression levels in B cells were significantly increased in patients with SLE compared to healthy subjects. In contrast, these increased expressions of TLR9 in B cells were not observed in the patients with RA, another autoimmune disease (Figures 4A, 4B). We evaluated the relationship between TLR9 expression on B cells and the treatment regimens in patients with SLE. However, there was no significant difference of TLR9 expression in the presence or absence of moderate doses of corticosteroids (PSL > 20 mg/day) or immunosuppressants (Figure 5). Similarly, there was no significant difference of TLR2 expression on CD14+ monocytes in the presence or absence of moderate doses of corticosteroids or immunosuppressants (Figure 6). We also evaluated the correlations of the TLR9 expression levels in B cells with clinical measures such as the SLEDAI, hemolytic complement (CH50), and titers of anti-dsDNA antibodies. However, there was no significant correlation between TLR9 expression levels and these clinical measures.

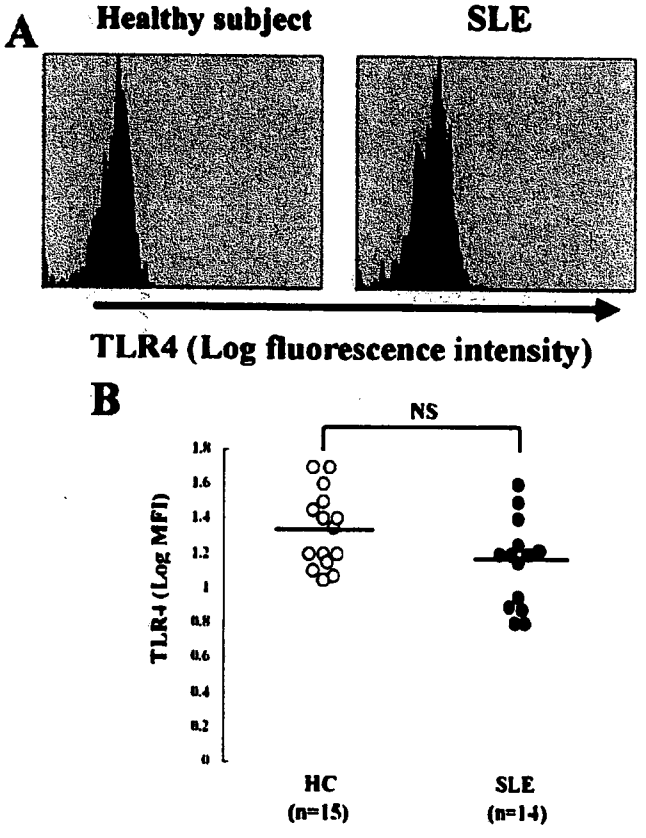


Figure 2. Expression of TLR4 on CD14+ monocytes in patients with SLE. A. Histograms show baseline expression of surface TLR4 on monocytes in a representative patient with SLE and healthy subject. Darker areas indicate staining with isotype control mAb. B. Mean fluorescence intensity of surface TLR2 staining for CD14+ monocytes in patients with SLE (n = 14) and healthy subjects (HC: n = 15). Significance of differences between groups were analyzed by Mann-Whitney U-test.

TLR9 expression in lupus PDC. It was demonstrated that TLR9 mediates the activation of PDC by SLE-IC. To investigate TLR9 expression on PDC, we performed double-staining of PBMC, with a mixture of mAb against CD123 and BDCA-2. PDC, identified as CD123/BDCA-2 double-positive populations[13], were gated (Figure 7) and analyzed for TLR9 expression. In order to make low proportions of peripheral blood PDC visible especially in patients with SLE, at least 100,000 cells were acquired. Figure 6A shows a representative result for the TLR9 expression of gated BDCA-2+, CD123+ cells from healthy subjects and patients with SLE. No significant difference between healthy subjects and patients with SLE was observed with regard to the TLR9 expression on PDC (Figure 8).

IgG production following CpG stimulation in B cells. To determine the ability of CpG ODN to stimulate immunoglobulin production by lupus B cells, T-cell-depleted MNC were cultured in the presence of CpG ODN. Cultured supernatants were harvested on Day 5 of culture and analyzed for the presence of IgG by ELISA. CpG ODN stimulated the production of IgG from control and lupus B cells. The increase in IgG production was 1.6-fold by control B cells, and an equivalent 1.9-fold increase in lupus B cells. This difference was not statistically significant. Under stimulation through BCR, the increase in IgG production was 5.6-fold in controls and 6.4-fold in SLE, and no significant difference was observed. These findings indicate that lupus B cells respond to CpG ODN stimulation; however, the ability to respond to CpG ODN in lupus B cells was not different from that in control B cells (Table 2, Figure 9).

DISCUSSION

Type 1 IFN has been proposed to have a pivotal etiopathogenic role in SLE, since serum levels of IFN-α correlate with the disease activity of SLE[5,6]. IFN-α may contribute to lupus autoimmune processes by differentiating B lymphocytes and following autoantibody production[14]. TLR are a family of pattern-recognition receptors that evolved to detect microbial infection[9]. These receptors recognize conserved molecular products derived from different classes of microorganisms[15]. Although TLR detect molecular patterns of microbial origin, some TLR and their ligands have emerged as important regulators of immunity relevant to effector responses to autoimmunity[16].

Recent data indicate that immune complexes containing

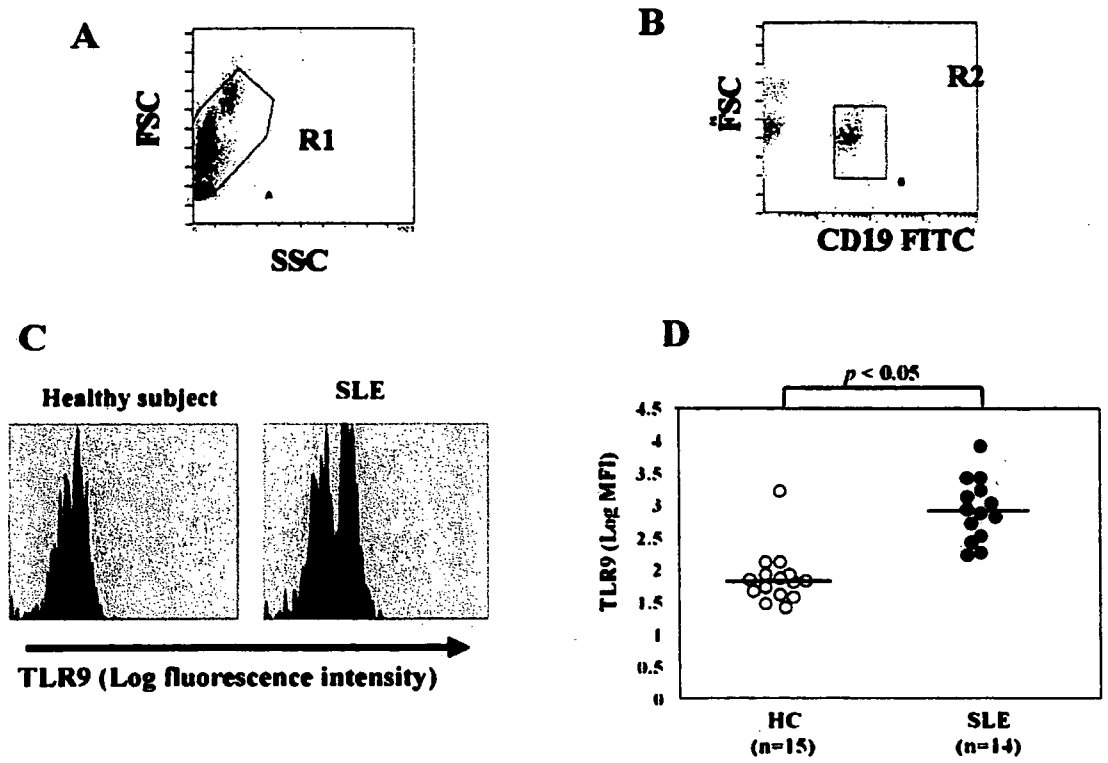
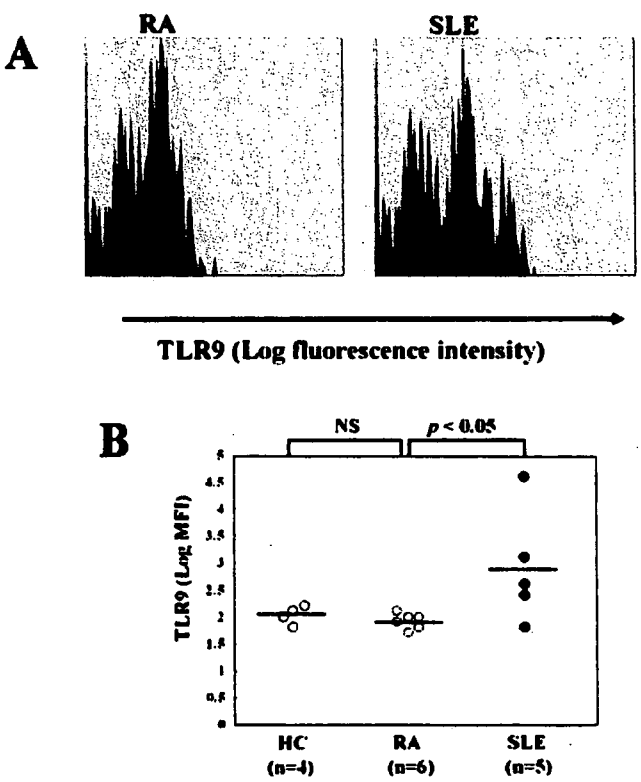


Figure 3. Upregulated expression of TLR9 on CD19+ B cells in patients with SLE. A, B. Isolation of CD19+ B cells by flow cytometry. PBMC were assessed by forward scatter (FS) and side scatter (SS) measures. Cells gated in R1 (A) were further analyzed for anti-CD19 FITC-labeled mAb. CD19+ cells were gated (R2) and defined as B cells (panel B). C. Histograms depicting baseline expression of intracellular TLR9 on B cells in a representative patient with SLE and healthy subject. Darker areas indicate staining with isotype control mAb. D. Mean fluorescence intensity of intracellular TLR9 staining for CD19+ B cells in patients with SLE (n = 14) and healthy subjects (HC; n = 15). Significance of differences between groups were analyzed by Mann-Whitney U-test.



nucleic acid and lupus IgG can induce IFN-α secretion from PDC[8]. Further, Means, *et al* showed that SLE-IC stimulates PDC to produce IFN-α in a TLR9-dependent manner[17]. The ability of PDC to secrete IFN-α depends on cellular sensors that detect the presence of bacterial DNA or IC[18], and TLR9 expression may account for the IFN-α production from PDC. We investigated the expression of TLR9 on circulating PDC in patients with SLE. Our data show that the expression of TLR9 in PDC was not different between patients with SLE and healthy subjects. Previous investigations showed that the numbers of circulating PDC are decreased in patients with SLE[19], but large numbers of activated PDC infiltrate skin lesions and actively produce IFN-α[20]. These findings suggest that PDC seem to be activated by IC in the pathogenic lesions and circulating PDC may not reflect these immune conditions. Further investigations concerning the TLR9 expression in tissue-infiltrating PDC are needed in patients with SLE.

Figure 4. Expression of TLR9 on CD19+ B cells in patients with RA and SLE. A. Histograms show baseline expression of intracellular TLR9 on B cells in representative patients. Darker areas indicate staining with isotype control mAb. B. Mean fluorescence intensity of intracellular TLR9 staining for CD19+ B cells in healthy subjects (HC, n = 4), patients with RA (n = 6), and with SLE (n = 5). Significance of differences between groups were analyzed by Mann-Whitney U-test.

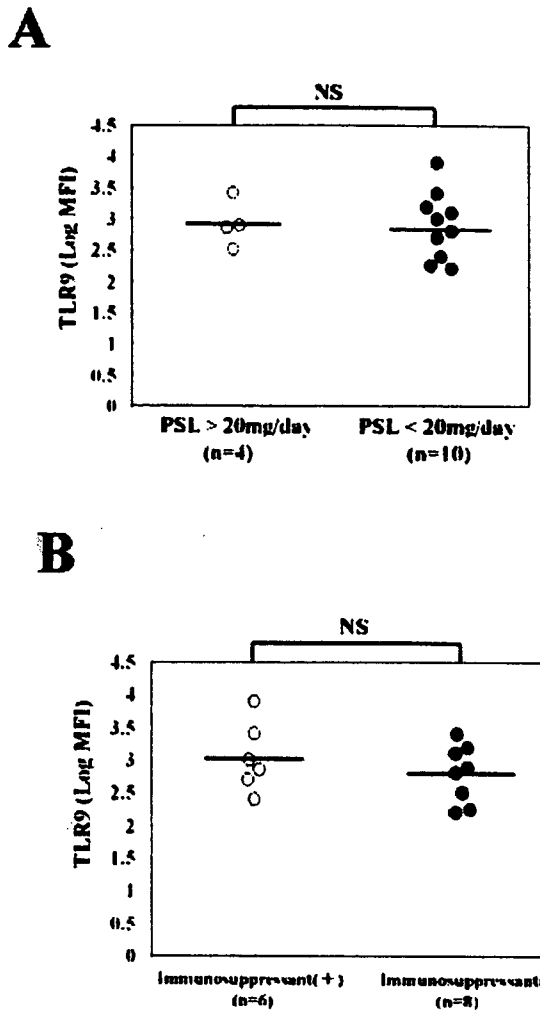


Figure 5. Expression of TLR9 on CD19+ B cells in patients with SLE. A. Mean fluorescence intensity (MFI) of intracellular TLR9 staining for CD19+ B cells in patients with SLE treated with > 20 mg/day prednisolone (PSL; n = 4) or < 20 mg/day PSL (n = 10). Significance of differences between groups were analyzed by Mann-Whitney U-test. B. MFI of intracellular TLR9 staining for CD19+ B cells in patients with SLE treated with (n = 6) or without immunosuppressants (n = 8). Significance of differences between groups were analyzed by Mann-Whitney U-test.

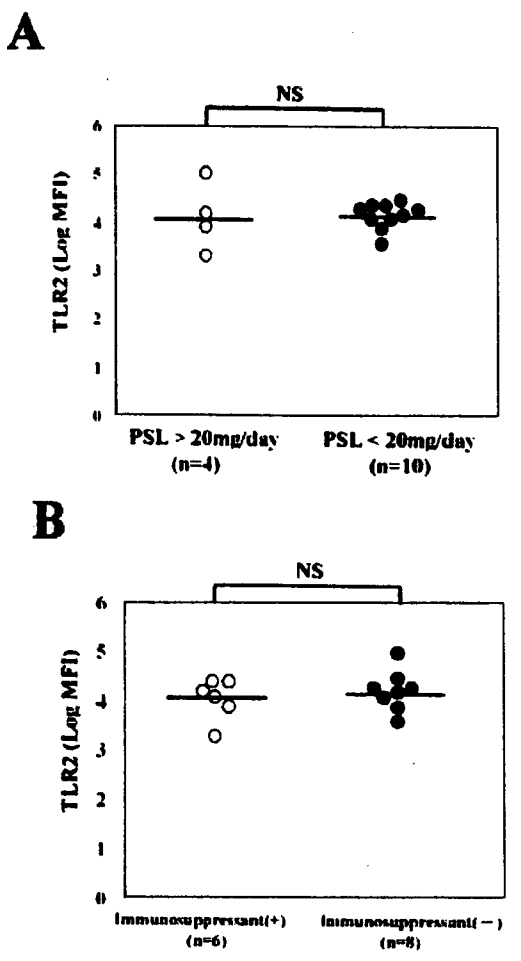


Figure 6. Expression of TLR2 on CD14+ monocytes in patients with SLE. A. Mean fluorescence intensity (MFI) of intracellular TLR2 staining for CD14+ B monocytes in patients with SLE treated with > 20 mg/day prednisolone (PSL; n = 4) or < 20 mg/day PSL (n = 10). Significance levels for differences between groups were analyzed by Mann-Whitney U-test. B. MFI of intracellular TLR2 staining for CD14+ monocytes in patients with SLE treated with (n = 6) or without immunosuppressants (n = 8). Significance of differences between groups were analyzed by Mann-Whitney U-test.

The high serum levels of IFN-α in patients with SLE were found to promote the differentiation of B cells as well as to activate PDC[21]. Similar to PDC, B cells express a limited set of TLR, including TLR7 and TLR9[22]. Leadbetter, *et al* showed that autoreactive B cells are activated by IgG-chromatin IC and require the synergistic engagement of B cell receptor and TLR9[23]. It is postulated that engagement of the BCR by an autoantibody-antoantigen IC triggers the endocytosis of IC that then results in the efficient delivery of chromatin fragments to endosome-associated TLR9[23]. We consistently observed the expression of TLR9 in freshly isolated human peripheral blood B cells, and our first major observation was the upregulated expression of TLR9 in peripheral blood B cells from patients with SLE. More recently, it was postulated that the engagement of TLR receptors, particularly TLR9, on B cells seems to play an important role in B cell activation and autoantibody production[24]. The importance of the TLR9-dependent pathways will depend on the levels of TLR9 expression. The differential expression of TLR9 may correlate with the responsiveness to CpG DNA, and the altered TLR9 expression could potentially affect the B cell immune response to chromatic or IC in patients with SLE.

CpG ODN have been shown to activate B cells via the family of TLR9[25]. Our data indicated that both control and lupus B cells responded to CpG DNA stimulation. The ability of lupus B cells to produce IgG was higher compared to control B cells; however, the difference was not significant. Since our B cell preparations contain mononuclear cells (T-cell-depleted mononuclear cells), the abnormal response of non-B cell pop-

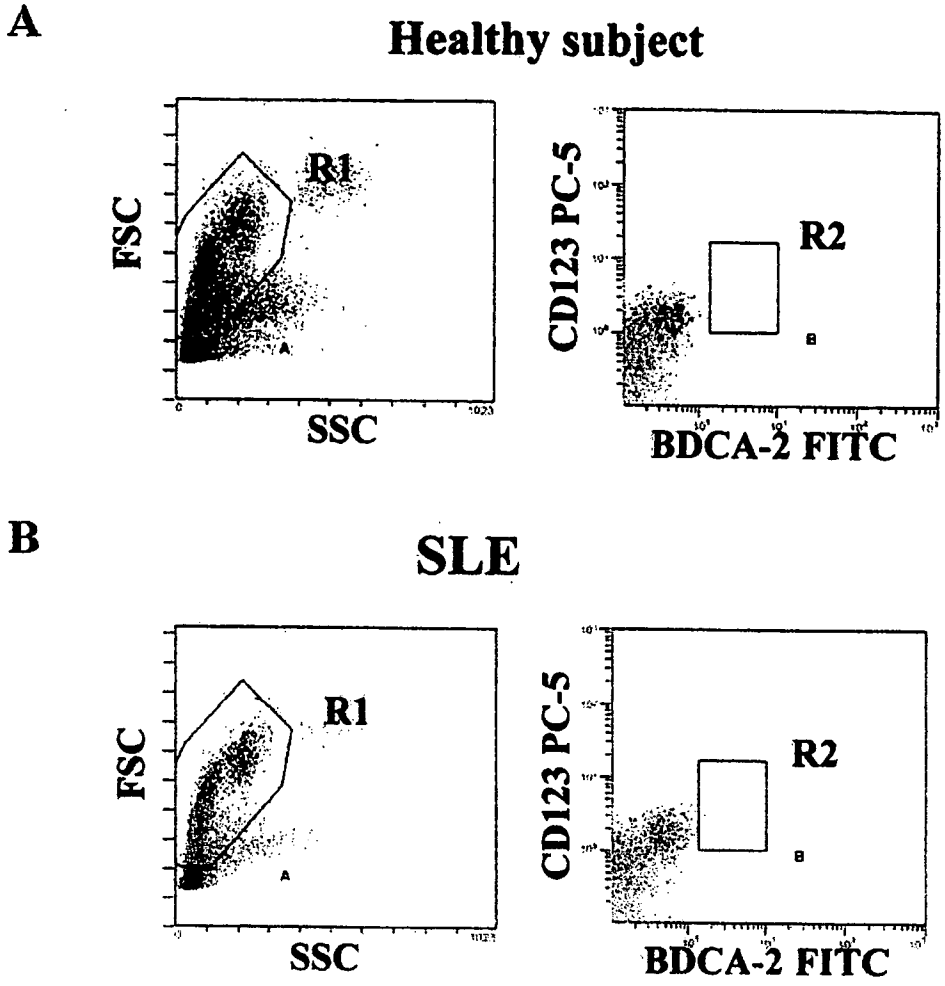


Figure 7. Identification of circulating plasmacytoid dendritic cells (PDC) by flow cytometry in peripheral blood from a representative patient with SLE and healthy subject. Mononuclear cells isolated from a healthy subject (A) and patient with SLE (B) were assessed by forward scatter (FS) and side scatter (SS). The R1-gated events were then analyzed for BDCA-2 and CD123, and BDCA-2+, CD-123+ double-positive cells (R2) were defined as PDC.

ulations to CpG DNA contributed to this phenomenon as described previously[26].

The exact immune mechanisms underlying the upregulation of TLR9 on lupus B cells remain to be elucidated in our study. Bernasconi, *et al* indicated that in human naive B cells, TLR9 is expressed at low to undetectable levels, but its expression is rapidly upregulated by BCR triggering[27]. In contrast, memory B cells expressed TLR9 at constrictively high levels[28]. SLE is characterized by polyclonal B cell activation[29], and these alternations of B cell activation or differentiation status may account for the upregulation of TLR9 expression. We could not show the interaction between increased TLR9 expression on B cells and lupus disease activity. Our study included 14 patients with SLE with low to moderate disease activity; therefore, further large-scale investigations of patients with SLE are needed to elucidate the relationship between TLR9 expression on B cells and lupus disease activity.

In summary, we showed that TLR9 expression in B lymphocytes was increased in patients with SLE. This upregulated TLR9 expression may activate B lymphocytes through the interaction between TLR9 and its ligands and may be related to the pathogenesis of lupus.

REFERENCES

1. Ruiz-Irastorza G, Khamashta MA, Castellino G, Hughes GR. Systemic lupus erythematosus. Lancet 2001;357:1027-32.
2. Hahn BH. Antibodies to DNA. N Engl J Med 1998;338:1359-68.
3. ter Borg EJ, Horst G, Hummel EJ, Limburg PC, Kallenberg CG. Measurement of increases in anti-double-stranded DNA antibody levels as a predictor of disease exacerbation in systemic lupus erythematosus. A long-term, prospective study. Arthritis Rheum 1990;33:634-43.
4. Ebling FM, Hahn BH. Pathogenic subsets of antibodies to DNA. Int Rev Immunol 1989;5:79-95.
5. Ronnblom L, Alm GV. An etiopathogenic role for the type I IFN system in SLE. Trends Immunol 2001;22:427-31.

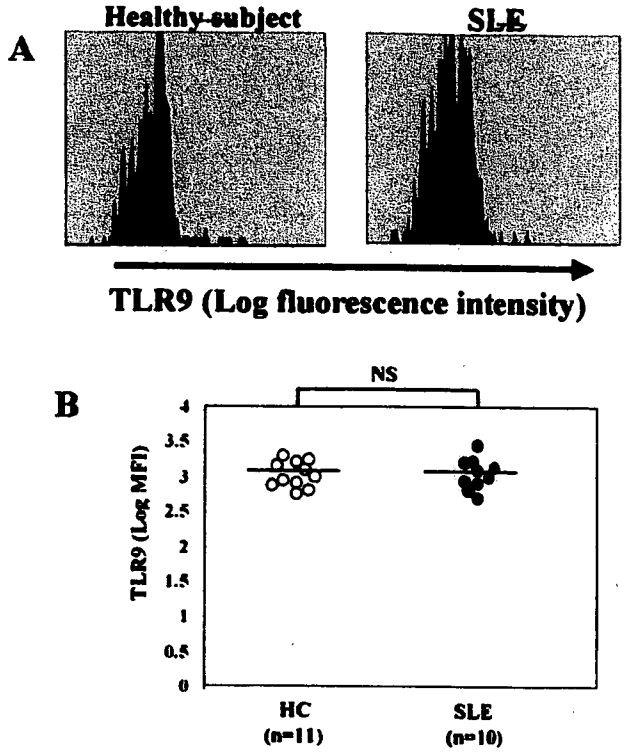


Figure 8. Expression of TLR9 on plasmacytoid dendritic cells (PDC) in patients with SLE. A. Histograms show baseline expression of intracellular TLR9 on PDC in a representative patient with SLE and healthy subject. Darker areas indicate staining with isotype control mAb. B. MFI of surface TLR9 staining for PDC in patients with SLE (n = 14) and healthy subjects (HC; n = 15). Significance of differences between groups were analyzed by Mann-Whitney U-test.

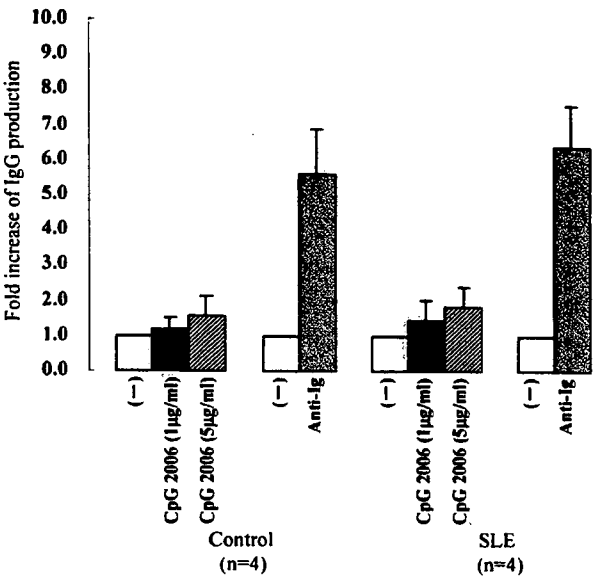


Figure 9. Secretion of IgG by PBMC from patients with SLE (n = 4) and healthy subjects (control; n = 4) after stimulation with CpG ODN. Freshly isolated T cell-depleted MNC were cultured with CpG ODN and anti-IgG. The amounts of IgG in culture supernatants were determined by ELISA. The IgG secreted from untreated MNC was assigned the value of 1.0 and data were expressed as fold induction. These data represent mean ± SD of 4 independent samples.

Table 2. Immunoglobulin production after 4 days of culture with CpG oligodeoxynucleotide. Results are mean (SD) of duplicate samples.

	IgG, ng/ml			
	None	CpG 2006 (1 μg/ml)	CpG 2006 (5 μg/ml)	Anti-Ig
Control				
1	125.2 (21.7)	135.1 (17.4)	212.6 (17.9)*	417.2 (44.7)*
2	106.2 (9.5)	155.5 (25.1)*	195.4 (32.1)*	517.2 (69.1)*
3	97.6 (11.6)	105.6 (9.9)	121.5 (17.8)*	617.5 (55.2)*
4	79.1 (14.8)	85.5 (13.2)	96.5 (9.5)	715.2 (69.9)*
SLE				
1	105.4 (7.2)	252.4 (19.2)*	289.2 (17.7)*	781.2 (91.1)*
2	132.4 (16.3)	167.5 (12.1)	245.1 (21.9)*	824.6 (102.9)*
3	108.8 (8.9)	118.2 (9.4)	162.5 (12.7)*	654.9 (78.1)*
4	152.7 (11.1)	191.9 (17.1)*	233.5 (29.1)*	932.7 (115.4)*

* $p < 0.05$ vs No stimulation.

6. Ronnblom L, Alm GV. Systemic lupus erythematosus and the type I interferon system. Arthritis Res Ther 2003;5:68-75.
7. Ronnblom L, Alm GV. A pivotal role for the natural interferon alpha-producing cells (plasmacytoid dendritic cells) in the pathogenesis of lupus. J Exp Med 2001;194:59-63.
8. Lovgren T, Eloranta ML, Bave U, Alm GV, Ronnblom L. Induction of interferon-alpha production in plasmacytoid dendritic cells by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG. Arthritis Rheum 2004;50:1861-72.
9. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003;21:335-76.
10. Pasare C, Medzhitov R. Toll-like receptors: linking innate and adaptive immunity. Adv Exp Med Biol 2005;560:11-8.
11. Tan EM, Cohen AS, Fries JF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271-7.
12. Urowitz MB, Gladman DD. Measures of disease activity and

damage in SLE. Baillieres Clin Rheumatol 1998;12:405-13.
13. Dzionek A, Fuchs A, Schmidt P, et al. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J Immunol 2000;165:6037-46.
14. Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, Tough DF. Type 1 interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. Immunity 2001;14:461-70.
15. Kopp E, Medzhitov R. Recognition of microbial infection by Toll-like receptors. Curr Opin Immunol 2003;15:396-401.
16. Rifkin IR, Leadbetter EA, Busconi L, Viglianti G, Marshak-Rothstein A. Toll-like receptors, endogenous ligands, and systemic autoimmune disease. Immunol Rev 2005;204:27-42.
17. Means TK, Latz E, Hayashi F, Murali MR, Golenbock DT, Luster AD. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. J Clin Invest 2005;115:407-17.
18. Bauer S, Wagner H. Bacterial CpG-DNA licenses TLR9. Curr Top Microbiol Immunol 2002;270:145-54.
19. Scheinecker C, Zwolfer B, Koller M, Manner G, Smolen JS. Alterations of dendritic cells in systemic lupus erythematosus: phenotypic and functional deficiencies. Arthritis Rheum 2001;44:856-65.
20. Farkas L, Beiske K, Lund-Johansen F, Brandtzaeg P, Jahnsen FL. Plasmacytoid dendritic cells (natural interferon-alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions. Am J Pathol 2001;159:237-43.
21. Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. Annu Rev Immunol 2005;23:275-306.
22. Hornung V, Rothenfusser S, Britsch S, et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol 2002;168:4531-7.
23. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature 2002;416:603-7.
24. Viglianti GA, Lau CM, Hanley TM, Miko BA, Shlomchik MJ, Marshak-Rothstein A. Activation of autoreactive B cells by CpG dsDNA. Immunity 2003;19:837-47.
25. Bauer S, Kirschning CJ, Hacker H, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci USA 2001;98:9237-42.
26. Zeuner RA, Klinman DM, Illei G, et al. Response of peripheral blood mononuclear cells from lupus patients to stimulation by CpG oligodeoxynucleotides. Rheumatology Oxford 2003;42:563-9.
27. Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood 2003;101:4500-4.
28. Kikuchi K, Lian ZX, Yang GX, et al. Bacterial CpG induces hyper-IgM production in CD27(+) memory B cells in primary biliary cirrhosis. Gastroenterology 2005;128:304-12.
29. Renaudineau Y, Pers JO, Bendaoud B, Jamin C, Youinou P. Dysfunctional B cells in systemic lupus erythematosus. Autoimmun Rev 2004;3:516-23.