The frequency and intensity of follow-up for patients with potentially curative surgical treatment varies considerably. The follow-up schedules published by several prominent medical centers for PSA tests and digital rectal examination (DRE) are every 2–3 months for the first year, and every 4–6 months thereafter.[5] However, surveys of American Urological Association (AUA) urologists suggest that for healthy patients who have undergone radical prostatectomy for stage T1 to T3aN0M0 prostate cancer follow-up should include an office visit with DRE, PSA, and urinalysis every 3 months in the first year, every 6 months in the second to fifth year, and annually thereafter.[4] Bone scan, abdominal and pelvic computerized tomography (CT) and magnetic resonance imaging (MRI), are rarely recommended for healthy patients.[2,4,5] These follow-up schedules are mainly for patients following radical prostatectomy without adjuvant hormone therapy. Few reports exist on the follow-up of patients after surgery with adjuvant androgen deprivation.

In the present review, we focus our attention on two questions concerning follow-up procedures after radical prostatectomy. First, is the determination of PSA levels a dependable indicator in patients who have undergone radical prostatectomy with adjuvant hormone therapy? Second, what is the appropriate follow-up procedure and frequency to minimize medical cost without missing signs of consequential recurrence and adversely affecting the patients?

Methods

Two hundred and twenty-one patients with clinical T1c, T2 and T3 prostate cancer who underwent a radical prostatectomy between June 1989 and May 1999, and were followed up at our hospital, were consecutively included in this retrospective study. The patients ranged from 48 to 84 (mean 61.3) years of age. Follow-up periods ranged from 0 to 144 (mean 46.3) months. Eighty percent of the patients in this series received neo-adjuvant hormone therapy for 3–12 (mean 5.8) months. Based on pathological results such as a high Gleason score, or high stage or positive lymph nodes, 177 patients also received continuous adjuvant hormone therapy after the operation[6] with the intention of prolonging the time before treatment failure.[3] Some patients had previously received neo-adjuvant hormone therapy at other institutions when they presented at our hospital. Consequently, initial PSA levels were not available.

After the operation, patients were followed up every 3–4 months by a PSA check (until 6.1997, Delfia PSA,[7] lower limit = 0.1 ng/mL; 7.1997 to 3.1999, Lumipulse PSA,[8] lower limit = 0.1 ng/mL; and after 3.1999, high sensitive Lumipulse PSA, lower limit = 0.03 ng/mL). Patients without detectable PSA levels were examined for recurrences annually by pelvic and abdominal CT, bone scan and chest X-ray. This occurred because 80.1% of the patients received continuous adjuvant hormone therapy, and PSA levels under hormonal regulation may not accurately reflect clinical findings.[9] Tumor recurrence was defined as biochemical failure when detectable PSA (> 0.1 ng/mL) was found in two consecutive assays, and/or as clinical failure when local recurrence or metastatic disease was detected. In the patients who exhibited biochemical failure, surveys of local recurrence by DRE and trans-rectal ultrasonography, as well as screening of distant metastasis by pelvic and abdominal CT, bone scan and chest X-ray were conducted.

To identify the ideal follow-up strategy after radical prostatectomy, pathological factors such as pT stage, status of surgical margin and Gleason's score were analyzed in relation to the risk of tumor recurrence in all patients, including patients with adjuvant hormone therapy. The type of examination that first demonstrated tumor recurrence and the findings of other examinations at that time were analyzed. To determine the appropriate follow-up interval, PSA level doubling times after biochemical failure were also analyzed.

The pT stage is classified according to the *TNM Classification of Malignant Tumors* (5th edn, 1997).[10] Surgical margin of the specimen was examined by 5-mm step sections transversely in the middle, and longitudinally in the apex and base of the prostate. Prostate specific antigen level doubling time was calculated by linear regression.[11] Welch's *t*-test and Wilcoxon's test were used for statistical analysis.

Results

Biochemical failure and clinical failure

Figure 1 shows the total number of patients without recurrence and the percentage of recurrence at each postoperative year. Tumor recurrence was identified in 30 patients (13.6%). Delayed recurrence (more than 5 years after operation) was detected in three patients (10%). These three patients received adjuvant endocrine therapy. In all 30 patients who experienced recurrence, biochemical failure was detected initially without clinical failure, despite that most of our patients underwent adjuvant hormone therapy. Of 30 patients with biochemical failure, six patients (20%) developed clinical

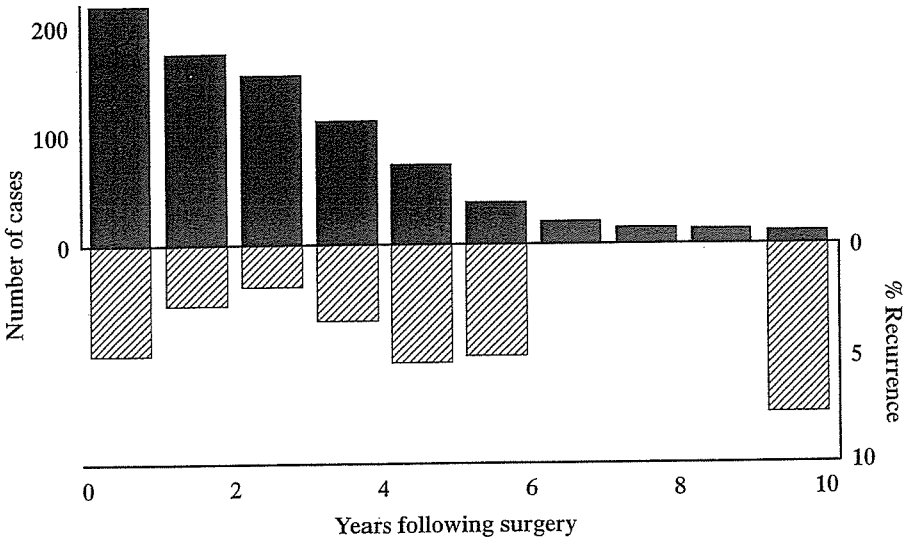


Fig. 1 Total number of prostate cancer patients without recurrence and percentage of recurrence at each postoperative year. ■, total number of patients without recurrence; ▨, percent recurrence.

Table 1 Pathological and clinical features in relation to recurrence

	Total cases			Cases with adjuvant hormone therapy		
	Number	Recurrence		Number	Recurrence	
		Number	%		Number	%
Stage						
pT2. pN0	78	10	12.8	59	2	3.4
pT3. pN0	92	11	12.0	70	5	7.1
pT4 or pTx. pN1	49	9	18.4	48	9	18.8
Surgical margin						
Negative	75	14	18.7	67	9	13.4
Positive	141	16	11.3	110	7	6.4
Gleason's sum						
6≧	66	14	21.2	47	4	8.5
7≦	154	16	10.4	130	12	9.2
Adjuvant Hx						
+	177	16	9.0			
−	42	14	33.3			

failure (one lung metastasis, two bone metastases and three pathologically defined local recurrences) with PSA levels of 0.6–28.1 ng/mL. The other 24 patients (80%) still display elevated PSA levels as the sole sign of recurrence. For six patients with clinically recurrent disease, 3–36 (mean 15.5) months were required to definitively identify clinical failure following the initial detection of elevated PSA levels. In this series, no patients with undetectable PSA levels developed recurrent disease that was detected as clinical failure.

Risk factors for tumor recurrence

Pathological findings and the status of adjuvant hormone therapy in 221 patients are summarized in relation to tumor recurrence in Table 1. There was no significant correlation between tumor recurrence and pT stage, and the presence or absence of a positive surgical margin or Gleason's score. Adaptation of adjuvant hormone therapy seems to have some benefit ($P=0.00018$). Pathological findings and clinical features of patients with recurrent disease who had either undergone adjuvant hormone therapy or not are summarized in Table 2. There are significant differences in the two groups with regard to Gleason's sum and the pathological stage of disease. There is no significant correlation between disease-free duration and the way recurrence was detected (biochemical failure or clinical failure).

Prostate specific antigen velocity and doubling time

Figure 2 shows PSA velocity after radical prostatectomy according to Gleason's' score (less than seven or more)

Table 2 Pathological and clinical features of patients with recurrent disease who had either undergone adjuvant hormone therapy or not

		With adjuvant hormone therapy	Without adjuvant hormone therapy	*P*-value
Age (average) (years)		64.1	61.5	0.280
Gleason's sum	6≧	4	10	0.020
(number of cases)	7≦	12	4	
Stage	pT2	2	8	0.0012
(number of cases)	pT3	5	6	
	pT4 or N1	9	0	
Surgical margin	Negative	7	9	0.34
(number of cases)	Positive	9	5	
Duration of PSA failure free (average, months)		37.9	21.6	0.099
Type of recurrence	PSA elevation alone	13	11	0.897
(number of cases)	Developed clinical failure	3	3	

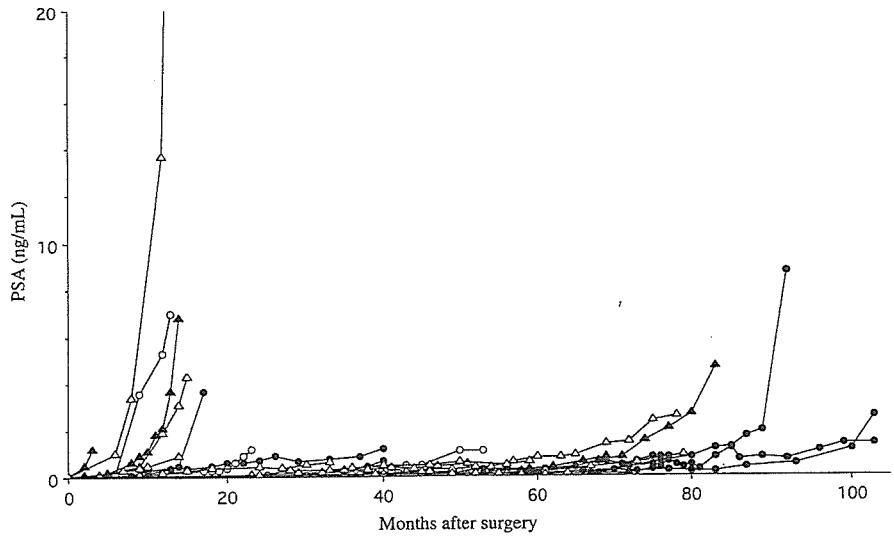


Fig. 2 Serum prostate specific antigen (PSA) after radical prostatectomy. ▲, Gleason's sum 7≤, with adjuvant hormone therapy; ●, Gleason's sum 6≥, with adjuvant hormone therapy; △, Gleason's sum 7≤, without adjuvant hormone therapy; and ○, Gleason's sum 6≥, without adjuvant hormone therapy.

and the status of adjuvant hormone therapy in patients who experienced recurrence. There was no definitive correlation between PSA velocity and these factors. Table 3 shows PSA doubling times for each patient group classified by Gleason's sum, with or without adjuvant hormone therapy, and the time to recurrence. In patients who displayed elevated PSA levels within 12 months after radical prostatectomy, PSA levels rapidly increased with a doubling time of 1.2–13.7 (mean 4.7, median 2.0) months. Excluding these cases, the doubling time of serum PSA levels was 2.8–31.5 (mean 10.6, median 9.0) months (two-tailed $P=0.019$).

Discussion

To determine the appropriate postoperative follow-up strategy for patients who undergo radical prostatectomy, we need to consider several issues, including the survival benefit of earlier detection of recurrence, the duration of follow-up, the examination procedures, and the frequency and intensity of follow-up.

The first issue is whether follow-up is even necessary. If earlier detection of treatment failure after radical prostatectomy provides no survival benefit, strict follow-up of patients may be of no use to the urologist or the patient. Whether early detection and early start of adjuvant therapy in patients with small amounts of residual cancer will control local disease or prolong survival is currently unknown.[12–14] Earlier detection of elevated PSA levels did, however, corresponded with smaller tumor volume. At this stage, residual cancer may be localized in the pelvic bed, and may respond more favorably to localized therapy with improved long-term survival.[12] However, the prophylactic use of early, as opposed to delayed adjuvant radiation therapy after radical prostatectomy showed no difference in treatment outcome.[15]

Table 3 Prostate specific antigen (PSA) doubling times after PSA failure

	Range	Average	Median	Standard deviation	Welch's *t*-test *P* (two-tailed)
Gleason's sum 6≧	1.56~31.5	9.55	7.30	8.64	0.503
Gleason's sum 7≦	1.22~13.72	7.64	6.57	4.66	
With adjuvant hormone therapy	1.93~13.72	7.58	7.00	4.38	0.491
Without adjuvant hormone therapy	1.22~31.5	9.46	7.61	8.54	
Recurred within 12 months of surgery	1.22~13.72	4.64	2.01	4.76	0.019
Recurred 12 months or more after surgery	2.78~31.5	10.57	8.98	6.79	
				Month	

The use of early, as opposed to delayed hormone therapy for rising PSA levels after radical prostatectomy is also a controversial issue.[13] For some patients with Gleason's score ≥8 and stage pT3b disease, early start of adjuvant therapy after radical prostatectomy provides survival benefits.[14] Experiences at Mayo Clinic[6,16] showed that early adjuvant androgen ablation therapy had a survival benefit for patients with pTxpN plus disease. In some instances, higher dose radiation therapy for pT3N0 prostate cancer showed durable efficacy when initiated at an early postoperative time.[17] Prostate specific antigen tests enabled the early detection of persistent or recurrent prostate cancer, but the survival benefits of early initiation of subsequent therapy are unknown for prostate cancer that is pathologically organ-confined or specimen-confined at the time of radical prostatectomy. Whether or not early detection provides a survival benefit depends on the outcome of large, randomized trials such as EORTC 30943 (immediate versus deferred hormonal treatment for PSA recurrence after definitive local treatment), and EORTIC 22911 (postoperative external radiotherapy versus no immediate further treatment in patients with pT3pN0 prostatic adenocarcinoma).

The second issue for discussion is the length of the follow-up period. Radical prostatectomy offers good clinical results for patients with organ-confined disease. However, the possibility of delayed treatment failure exists, especially in patients who received adjuvant hormone therapy or radiation therapy, and patients with a low-grade tumor. It was reported that 25% of recurred patients had an undetectable serum PSA level at 5 years of follow-up but progression to biochemical and local disease recurrence at a later time.[2] In our study, three of 30 patients (10%) experienced recurrence after five years or more. Some pathological and clinical factors (i.e. initial PSA level, PSA velocity, nadir PSA, Gleason's score, pT stage and seminal vesicle involvement) predict the risk of treatment failure[18–19] and distinguish local recurrence from distant metastases.[3] However, these factors are not ultimate predictors for a complete lack of tumor recurrence. Our data suggest no significant correlation between tumor recurrence and pathological factors. This may be due to the application of postoperative androgen deprivation or the definition of tumor recurrence. The long-term benefit of postoperative surveillance and the value of the various types of therapy for recurrence is currently unclear.[4] Urologists are therefore forced to continue follow-up of patients for a long time.

The third issue relates to types of postoperative examinations that should be performed. Determination of PSA levels is well documented as the most important indicator of tumor recurrence after radical prostatectomy without adjuvant androgen deprivation.[2,20] Theoretically, the serum PSA should drop to undetectable levels, even when determined by an ultrasensitive assay for PSA.[12] After radical prostatectomy, PSA levels of 0.4 ng/mL or more, strongly suggest the presence of residual disease[5] and the probable development of subsequent local recurrence or distant metastasis.

Clinical failure without biochemical failure is rare and sporadic, irrespective of whether patients have received concomitant adjuvant hormone therapy.[2,6,21] A survey of AUA urologists[4] suggests that bone scan, CT and MRI should not be recommended for healthy patients with undetectable PSA levels, considering the cost and rare incidence of recurrence. The risk of clinical failure in the absence of detectable PSA has been reported in cases with very high grade or anaplastic

tumors.[5] In our series, no clinical failure with undetectable PSA levels was observed and follow-up by PSA tests was shown to be reliable for the early detection of treatment failure, even for patients under androgen deprivation. The interval to clinical detection of treatment failure from the initial elevation of postoperative PSA required several (3–36) months. Therefore PSA tests are the most powerful indicator of recurrence in patients after radical prostatectomy with adjuvant hormone therapy.

The final issue for discussion is the ideal frequency and intensity of follow-up. To define the ideal timing of PSA checks, the doubling time of PSA levels was analyzed. Prostate specific antigen doubling time was not significantly different between patients with or without adjuvant hormone therapy. The PSA doubling time correlated with earlier versus delayed detection of biochemical failure, but not with pathological results or the status of adjuvant hormone therapy. It has been reported that it is difficult to predict PSA doubling time in an individual with relapse after radical prostatectomy.[21] Generally, a PSA level of 0.4 ng/dL or more after radical prostatectomy suggests the presence of residual disease.[5] In our series, the lowest PSA level was 0.6 ng/mL when a local recurrence or remote metastasis was detected. The minimum PSA doubling time was 1.2 months when biochemical failure was detected within 1 year after the operation, and 2.8 months when it was detected after the first year.

To avoid overlooking the signs of clinical failure, at least 0.4 ng/mL PSA level should be detected. Based on the calculated PSA doubling time, the longest appropriate interval between PSA checks can be estimated as every 2.4 months within the first postoperative year, and every 5.6 months thereafter if the lower limit of the PSA test kit used is 0.1 ng/mL; and every 4.6 months for the first year, and every 10.4 months thereafter if a highly sensitive PSA test kit (lower limit = 0.03 ng/mL) is used.

In conclusion, PSA is the most powerful indicator of treatment failure for patients who received radical prostatectomy with or without adjuvant hormone therapy. The use of CT, bone scan and MRI should be extremely limited in patients with undetectable PSA and no clinical symptoms. Our data indicates that, from a medical and economic perspective, check-ups including PSA testing to detect signs of clinical failure should occur every 4 months within the first postoperative year and biannually or annually thereafter.

In the present series, 191 of 221 patients who showed negative PSA and no clinical failure nevertheless received CT and bone scans. In Japan, a single PSA assay costs approximate US$45, an abdominal enhanced CT scan costs US$184, and a bone scan costs US$295. Consequently, in the first year US$479 dollars for each patient, and a total of 191 × US$479 dollars were wasted by unnecessarily intensive follow-up examinations.

References

1 Kakizoe T. Prostate carcinoma. In: Johnson FE, Virgo KS, eds. *Cancer Patients Follow-Up*. Mosby-Year Book, St Louis, 1997; 395–400.
2 Pound CR, Christens-Barry OW, Gurganus RT, Partin AW, Walsh PC. Digital rectal examination and imaging studies are unnecessary in men with undetectable prostate specific antigen following radical prostatectomy. *J. Urol.* 1999; **162**: 1337–40.
3 Partin AW, Pearson JD, Landis PK *et al.* Evaluation of serum prostate-specific antigen velocity after radical prostatectomy to distinguish local recurrence from distant metastases. *Urology* 1994; **43**: 649–59.
4 Joseph OH, John WC, David K *et al.* Current follow up strategies after radical prostatectomy: A survey of American Urological Association Urologists. *J. Uorl* 1999; **161**: 520–3.
5 Huben RP. Prostate carcinoma. In: Johnson FE, Virgo KS, eds. *Cancer Patients Follow-Up*. Mosby-Year Book, St Louis, 1997; 378–89.
6 Zinke H. Extended experience with surgical treatment of stage D1 adenocarcinoma of prostate: Significant influences of immediate adjuvant hormonal treatment on outcome. *Urology* 1989; **33**: 27–36.
7 Akimoto S, Akakura K, Ohki T *et al.* [Usefulness of hyper sensitive PSA assay kits for determination on low range of prostate specific antigen in prostate cancer.] *Jpn. J. Urol* 1995; **86**: 256–62 (in Japanese).
8 Nodate K, Tukada T. [Evaluation of chemiluminescent enzyme immunoassay 'Lumipulse PSA'.] *Jpn. J. Medical Pharm. Sci.* 1996; **36**: 1395–402 (in Japanese).
9 Leo ME, Bilhartz DL, Bergstralh EJ, Oesterling JE. Prostate specific antigen in hormonally treated stage D2 prostate cancer: Is it always an accurate indicator of disease status? *J. Urol.* 1991; **145**: 802–6.
10 Sobin LH, Wikind Ch (eds). ULCC TNM *Classification of Malignant Tumors*, 5th Edition, A John Wiley & Sons, 1997; 170–3.
11 Egawa S, Matsumoto K, Suyama K, Iwamura M, Kuwao S, Baba S. Observations of prostate specific antigen doubling time in Japanese patient with non-metastatic prostate carcinoma. *Cancer* 1999; **86**: 463–9.
12 Stamey TA, Graves HCB, Wehner N, Ferrari M, Freiha FS. Early detection of residual prostate cancer after radical prostatectomy by an ultrasensitive assay for prostate specific antigen. *J. Urol.* 1993; **149**: 787–92.
13 Bhayani SB, Andriole GL. Hormonal manipulation for rising PSA after radical prostatectomy. *Semin. Urol. Oncol.* 1999; **17**: 148–53.
14 Ruffion A, Valignat C, Champetier D, Lopez JG, Perrin P. Observation / delayed treatment for rising PSA after

radical prostatectomy. *Pros Cons. Semin. Urol. Oncol.* 1999; **17**: 135–40.
15 McCarthy JF, Catalona WJ, Hudson MA. Effect of radiation therapy on detectable serum prostate specific antigen levels following radical prostatectomy: Early versus delayed treatment. *J. Urol.* 1994; **152**: 1786–91.
16 Seay TM, Blute ML, Zincke H. Long-term outcome in patients with pTxN+ adenocarcinoma of prostate treated with radical prostatectomy and early androgen ablation. *J. Urol.* 1998; **159**: 357–64.
17 Valicenti RK, Gomella LG, Ismail M *et al.* Durable efficacy of early postoperative radiation therapy for high-risk pT3N0 prostate cancer: The importance of radiation dose. *Urology* 1998; **52**: 1034–40.
18 D'Amico AV, Whittington R, Malkowicz SB *et al.* The combination of preoperative prostate specific antigen and postoperative pathological findings to predict prostate specific antigen outcome in clinically localized prostate cancer. *J. Urol.* 1998; **160**: 2096–101.
19 D'Amico AV, Desjardin A, Chung A *et al.* Assessment of outcome prediction models for patients with localized prostate carcinoma managed with radical prostatectomy or external beam radiation therapy. *Cancer* 1998; **82**: 1887–96.
20 Trapasso JG, deKernion JB, Smith RB, Dorey F. The incidence and significance of detectable levels of serum prostate specific antigen after radical prostatectomy. *J. Urol.* 1994; **152**: 1821–5.
21 Oefelein MG, Smith N, Carter M, Dalton D, Schaeffer A. The incidence of prostate cancer progression with undetectable serum prostate specific antigen in a series of 394 radical prostatectomies. *J. Urol.* 1995; **154**: 2128–31.
22 Rabbani F, Perrotti M, Baster A, Fair WR. Prostate specific antigen doubling time after radical prostatectomy. Effect of Neoadjuvant androgen deprivation therapy. *J. Urol.* 1999; **161**: 847–52.

Jpn. J. Cancer Res. 93, 1063–1069, September 2002

Dose-response Trial of Lactoferrin in Patients with Chronic Hepatitis C

Shuichi Okada,[1] Katsuaki Tanaka,[5] Tosiya Sato,[6] Hideki Ueno,[1] Satoru Saito,[5] Takuji Okusaka,[1] Keiko Sato,[2] Seiichiro Yamamoto[3] and Tadao Kakizoe[4]

[1]Hepatobiliary and Pancreatic Oncology Division, [2]Clinical Research Coordinating Office, National Cancer Center Hospital, [3]Cancer Information and Epidemiology Division, National Cancer Center Research Institute, [4]National Cancer Center, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, [5]Gastroenterological Center, Yokohama City University Medical Center, 4-5 Urafune-cho, Minami-ku, Yokohama 232-0024 and [6]Department of Epidemiology and Biostatistics, Kyoto University School of Public Health, Yoshidakonoe-cho, Sakyo-ku, Kyoto 606-8501

Hepatitis C virus (HCV) is one of the most common causes of chronic hepatitis. Interferon is presently the only effective treatment for chronic hepatitis C (CH-C), though its effectiveness is limited. Lactoferrin (LF), which is an 80-kDa, iron-binding glycoprotein, has several biological activities including anti-viral activity, and it was recently reported to inhibit HCV infection in cultured human hepatocytes. The present trial was designed to assess the relationship between the dose of bovine LF (bLF) and the effect of bLF on serum alanine aminotransaminase (ALT) and HCV RNA levels in patients with CH-C. Forty-five patients entered at each of the three dose levels (bLF of 1.8, 3.6, and 7.2 g/day) received orally an 8-week course of bLF. There was no significant relation between the dose of bLF and the effect of bLF on serum ALT or HCV RNA levels. Biochemical (a 50% or greater decrease in the serum ALT level) and virological (a 50% or greater decrease in HCV RNA level) responses were observed in two and four patients, respectively, but all responders relapsed during the follow-up period after bLF treatment. The bLF treatment was generally well tolerated, and no patient had any serious adverse event. In conclusion, the excellent tolerance and potential anti-HCV activity of bLF shown in this trial suggest that further trials using a large number of patients are mandatory. We are currently conducting a double-blind randomized controlled trial comparing bLF with placebo to clarify the anti-HCV activity of bLF in patients with CH-C.

Key words: Lactoferrin — Chronic hepatitis C — Hepatitis C virus — Alanine aminotransaminase

Hepatitis C virus (HCV) is one of the most common causes of chronic hepatitis, and is also a major risk factor for hepatocellular carcinoma (HCC).[1)] Chronic hepatitis C (CH-C) is often asymptomatic and mild, but may slowly progress to liver cirrhosis and eventually to HCC.[2,3)] Therefore, treatment of CH-C and prevention of its complications assume utmost importance. At present, interferon (IFN) is the only effective treatment for CH-C, though its effectiveness is limited; a sustained biochemical (normalization of the serum alanine aminotransaminase (ALT) level) and virological (loss of detectable serum HCV RNA) response with histological improvement occurs in only 15–30% of CH-C patients.[4,5)] Moreover, adverse effects of IFN are sometimes severe enough to warrant cessation of treatment. To overcome these drawbacks of IFN, new formulations of IFN or combinations of IFN with ribavirin have been tested and have steadily improved sustained responses to treatment.[6–10)] Nevertheless, further developments in the treatment of CH-C are still needed to improve the prognosis of CH-C. The new agents against HCV may offer options to patients unable to take IFN or unable to achieve a response to IFN.

E-mail: sokada@ncc.go.jp

Lactoferrin (LF) is an 80-kDa, iron-binding glycoprotein present mainly in external secretions, such as breast milk, and in the secondary granules of neutrophils.[11,12)] Several biological activities of LF have been addressed, including regulation of iron homeostasis, primary nonspecific defense against pathogenic microorganisms, modulation of immunoreaction, and bactericidal and anti-viral effects. To date, it has been reported that LF has an antiviral effect against human herpes simplex virus-1, human immunodeficiency virus-1, and human cytomegalovirus.[13–16)] Moreover, LF was recently reported to markedly inhibit HCV infection in cultured human hepatocytes regardless of HCV strain.[17–19)] In addition, a recent clinical pilot study reported that treatment with bovine LF (bLF) is associated with a marked decrease in serum HCV RNA and/or ALT levels in some patients with CH-C.[20)]

Thus, bLF is one potential candidate for an anti-HCV reagent that may be effective for the treatment of patients with CH-C. bLF has the clinical advantage of a low risk of severe adverse effects, because it is a natural milk protein. The present dose-response trial was designed to assess the relationship between the dose of bLF and the effect of bLF on serum ALT and HCV RNA levels in patients with CH-C.

PATIENTS AND METHODS

Patients Patients eligible for study entry had pathologically confirmed CH-C. Each patient was required to meet the following eligibility criteria: 20–74 years of age; liver biopsy performed within 6 months before entry; a serum ALT level ≥twice the upper normal limit for at least 6 months; positivity for anti-HCV antibody by enzyme-linked immunosorbent assay test; an HCV RNA level of 1–1000 Kcopies/ml; no evidence of HCC on the basis of ultrasonography and/or computed tomography (CT) performed within 3 months before entry; adequate renal function [normal serum creatinine and blood urea nitrogen levels], liver function [total bilirubin level ≤2 mg/dl, serum albumin level ≥3 g/dl, and serum transaminases (ALT) levels ≤200 IU/liter], and bone marrow reserve [white blood cell count ≥2000/mm^3, platelet count ≥50 000/mm^3, and hemoglobin level ≥9 g/dl]; written informed consent.

The exclusion criteria were as follows: positivity for hepatitis B surface antigen; IFN therapy within 6 months before entry; immunomodulatory or corticosteroid therapy within 3 months before entry; intravenous glycyrrhizin treatment within 1 month before entry; pleural or pericardial effusion which is difficult to control; gastrointestinal bleeding; pregnant or lactating females; females of childbearing age unless using effective contraception; other serious medical conditions (e.g. active infection, severe cardiac or pulmonary disease, and psychiatric disorders).

Methods bLF tablets (450 mg/tablet), provided by Morinaga Milk Industry Co. (Tokyo), were administered orally twice or three times a day. Patients received an 8-week course of bLF on an outpatient basis and then were followed for the next 8 weeks. During the treatment, patients maintained a daily journal to record bLF intake and any adverse events experienced. Cohorts of 15 patients were entered at each of the three dose levels (bLF of 1.8, 3.6, and 7.2 g/day). The starting dose of bLF (1.8 g/day) was determined according to the results of the pilot study.[20] The first 15 were entered at the bLF dose of 1.8 g/day, and dose escalation to the next cohort was decided after evaluation by a safety review board of all the safety data collected on the first five patients who had completed the study period of 16 weeks. The safety review board consisted of two hepatologists (S. O. and K. T.) and one biostatistician (T. S.).

The protocol permitted dose modification (a 50% reduction in the assigned dose) for patients who had clinically significant adverse events or abnormalities in laboratory values. Patients were withdrawn from the study when they experienced adverse effects such as grade 4 hematological toxicity, total bilirubin level ≥3 mg/dl, serum transaminases (ALT) levels ≥300 IU/liter, serum creatinine ≥1.5 times the upper normal limit, or other grade 3 non-hematological toxicities. Patients were also removed from the study if it was difficult to continue bLF treatment because of the development of serious complications or if an investigator was concerned about their safety. Other reasons for withdrawal included a request to withdraw.

Pretreatment evaluation included a complete history and physical examination. In addition, a complete blood count, biochemistry tests, urinalysis, and serum tests were performed. All patients were assessed for 16 weeks in an outpatient setting for safety, tolerance, and efficacy of the 3 dose levels. Adverse events that were observed by the investigators or reported by the patients during the study period were recorded and graded for severity according to the Japan Society for Cancer Therapy criteria, which are fundamentally similar to the World Health Organization criteria and NCI Common Toxicity criteria.[21,22] The analysis of safety included all the patients who received at least one dose of bLF.

Efficacy was assessed by measurements of serum HCV RNA and ALT levels. These levels were measured on day 1; during treatment at weeks 2, 4, and 8; and after treatment at weeks 4 and 8. Serum HCV RNA levels were measured by an Amplicor HCV Monitor (Nihon Roche, Tokyo) with a sensitivity of 1 Kcopy/ml. A biochemical response was defined as a 50% or greater decrease in the serum ALT level compared with the baseline when the post-treatment value was ≤twice the upper normal limit. A virological response was a 50% or greater decrease in the HCV RNA level compared with the baseline. Responses were measured at the end of treatment. The duration of both a biochemical response and a virological response was calculated from the first day of treatment until relapse to the pretreatment baseline.

HCV serotypes were determined by a serotyping assay according to the method of Tsukiyama-Kohara *et al.* and Tanaka *et al.*[23,24] In this analysis, HCV serotype 1 corresponds to genotypes 1a and 1b of Simmonds classification, and HCV serotype 2 corresponds to genotypes 2a and 2b.[25] A single pathologist who was unaware of the patient identification, dose level, or treatment response interpreted liver biopsy samples taken before treatment. The degree of hepatic fibrosis was scored using the METAVIR system.[26] The protocol did not require repetitive biopsies.

The primary endpoint of this trial was the changes in the serum ALT levels, and the secondary endpoints were the changes in the serum HCV RNA levels, virological and biochemical responses at the end of treatment, and safety. Analyses were conducted on all 45 patients for the changes in serum ALT levels and the biochemical responses. Since the pretreatment HCV RNA level decreased to an undetectable level in one patient (bLF of 3.6 g/day), the analyses of the change in serum HCV RNA levels and the virological responses were conducted on 44 patients.

Table I. Baseline Characteristics of Patients According to bLF Dose Group

	Dose of bLF		
	1.8 g/day	3.6 g/day	7.2 g/day
No. of patients	15	15	15
Age[a] (years)	62 (54–73)	62 (38–71)	55 (37–74)
Gender (male/female)	6/9	9/6	11/4
History of IFN therapy	4 (27%)	6 (40%)	6 (40%)
ALT level[a] (IU/liter)	82 (52–270)	107 (51–375)	99 (52–168)
HCV RNA level[a] (Kcopies/ml)	341 (35–1492)	224 (<1–1354)	138 (3–1460)
HCV RNA serotype (1/2/ND[b])	13/2/0	10/3/2	11/3/1
Hepatic fibrosis (F0,1/F2–4)[c]	7/8	7/8	8/7

a) Median (range).
b) ND, not determined.
c) F, fibrosis (F0, no fibrosis, F1, portal fibrosis without septa, F2, portal fibrosis with rare septa, F3, numerous septa without cirrhosis, and F4, cirrhosis).

A linear dose-response was tested for the changes in the logarithm of serum ALT level and the changes in the logarithm of serum HCV RNA level using a linear regression model. The *P*-values calculated were one-sided, because only the decreasing trend of these two endpoints with an increase in bLF levels was of interest; the one-sided α level of significance was set at 0.05. A sample size of 15 patients per bLF dose was calculated to provide about 90% power (using a one-sided 0.05 level of significance) to detect a 50% reduction in the serum ALT level at the end of treatment in the bLF 7.2 g/day group. In addition to the above planned analyses, evaluations of the changes in serum HCV RNA levels at weeks 4 and 8 after the end of treatment were conducted. Since these were originally unplanned analyses, they were considered as exploratory. Analyses were performed by JMP 4.0 and PC SAS Release 6.02 (SAS Institute, Inc., Cary, NC).

The study was approved by the investigational review board of each participating institution according to the Declaration of Helsinki, and all the patients provided written informed consent.

RESULTS

Patients and treatment Forty-five patients were entered into this trial at three dose levels (15 patients at each level) from the two hospitals between March, 1999 and May, 2000. There were 26 males and 19 females with a median age of 62 years (range, 38–74). Characteristics of the patients at baseline are summarized in Table I. There were no significant differences between the three dose levels with respect to age, gender, history of IFN therapy, baseline ALT levels, baseline viral characteristics, or hepatic fibrosis. Of the 45 patients enrolled in the study, 16 (36%) had previously received IFN treatment, but had had no sustained virological response. Serum ALT levels on day 1 ranged from 52 to 375 IU/liter (median, 99 IU/liter). Serum HCV RNA levels on day 1 ranged from ≤1 to 1492 Kcopy/ml (median, 227 Kcopy/ml), and the majority of the patients (76%) were infected with HCV serotype 1.

Table II. Biochemical and Virological Responses at the End of Treatment According to bLF Dose Group

	Dose of bLF		
	1.8 g/day (%)	3.6 g/day (%)	7.2 g/day (%)
Biochemical response	1/15 (7)	1/15 (7)	0/15 (0)
Virological response	4/15 (27)	0/14 (0)	0/15 (0)

Efficacy There was no significant relation between the dose of bLF and the effect of bLF on serum ALT (P=0.30) or HCV RNA levels (P=0.20) at the end of treatment; the patients at each level did not significantly differ with regard to the changes in serum ALT or HCV RNA levels.

Table II summarizes the biochemical and virological responses at the three dose levels at the end of treatment. Biochemical response was observed in two patients (bLF of 1.8 g/day and 3.6 g/day) at the completion of treatment, but no patient achieved ALT normalization. Virological response was observed in four patients (bLF of 1.8 g/day) at the end of treatment, although all had persistently detectable serum HCV RNA levels. All patients who responded to bLF relapsed during the follow-up period after bLF treatment; the durations of the biochemical responses were 5 and 6 months, and the durations of the virological responses ranged from 6 to 10 months. In some patients, serum HCV RNA levels were reduced even after the completion of treatment; the virological response was observed in seven patients at week 4 and eight patients at week 8 after the end of treatment.

In the virological responders, the reduction in HCV RNA levels, which was rapid after the initiation of treatment, was invariably associated with a decrease in serum ALT levels. The effects of bLF on serum HCV RNA and ALT levels in one representative patient from the study are shown in Fig. 1. Although the decrease in serum HCV RNA levels preceded the decrease in serum ALT levels, the time course of serum HCV RNA levels paralleled serum ALT levels during and after bLF treatment.

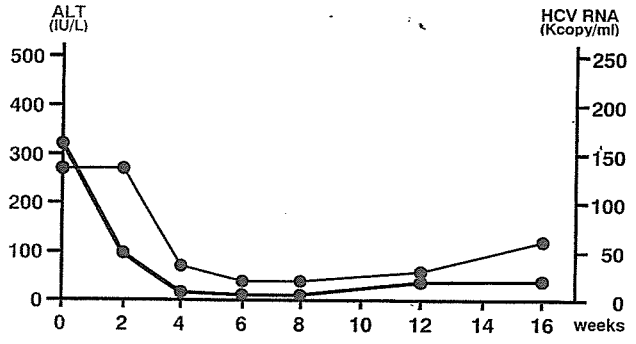


Fig. 1. Time course of changes in serum ALT and HCV RNA levels during and after bLF (1.8 g/day) treatment in a 71-year-old woman with chronic hepatitis C. —— ALT, —— HCV RNA.

The efficacy of bLF treatment was analyzed with respect to several baseline parameters. Table III shows the virological response rates at week 8 after the completion of treatment for these variables. Age was associated with the response to bLF treatment; in contrast to IFN therapy, the response rate was significantly higher among the patients of 60 years or older, although the reason for these inconsistent results remains unclear. However, the virological response was not significantly influenced by gender, a history of IFN therapy, granulocyte count, serum ALT levels, or hepatic fibrosis. The virological response was more common in patients with serum HCV RNA levels of less than 100 Kcopies/ml, compared with patients with levels equal to or greater than 100 Kcopies/ml, but there was no significant difference. Similarly, the response rate was somewhat higher in patients with HCV serotype 2, com-

Table III. Virological Response at Week 8 after the Completion of bLF Treatment with Respect to Baseline Characteristics of Patients

	Dose of bLF			
	1.8 g/day (%)	3.6 g/day (%)	7.2 g/day (%)	All doses (%)
Age*				
−59 years	0/4 (0)	0/7 (0)	0/8 (0)	0/19 (0)
60 years−	5/11 (45)	1/7 (14)	2/7 (29)	8/25 (32)
Gender				
Male	2/6 (33)	0/9 (0)	2/11 (18)	4/26 (15)
Female	3/9 (33)	1/5 (20)	0/4 (0)	4/18 (22)
History of IFN				
+	1/4 (25)	0/6 (0)	0/6 (0)	1/16 (6)
−	4/11 (36)	1/8 (13)	2/9 (22)	7/28 (25)
Granulocyte count				
$-1.9\times10^3/mm^3$	2/3 (67)	1/4 (25)	0/5 (0)	3/12 (25)
$2.0\times10^3/mm^3-$	3/12 (25)	0/10 (0)	2/10 (20)	5/32 (17)
ALT level				
−99 IU/liter	3/8 (38)	0/6 (0)	1/8 (13)	4/22 (18)
100 IU/liter−	2/7 (29)	1/8 (13)	1/7 (14)	4/22 (18)
HCV RNA level				
−99 Kcopies/ml	1/2 (50)	1/4 (25)	2/5 (40)	4/11 (36)
100 Kcopies/ml−	4/13 (31)	0/10 (0)	0/10 (0)	4/33 (12)
HCV RNA serotype				
1	4/13 (31)	0/10 (0)	1/11 (9)	5/34 (15)
2	1/2 (50)	1/2 (50)	1/3 (33)	3/7 (43)
Hepatic fibrosis[a)]				
F0, 1	2/7 (29)	0/6 (0)	1/8 (13)	3/21 (14)
F2–4	3/8 (38)	1/8 (13)	1/7 (14)	5/23 (22)

a) F, fibrosis (F0, no fibrosis, F1, portal fibrosis without septa, F2, portal fibrosis with rare septa, F3, numerous septa without cirrhosis, and F4, cirrhosis).
* $P<0.01$.

Table IV. Adverse Effects of bLF Treatment for CH-C

Case	Dose of bLF	Adverse effect (grade)	Dose reduction
1	3.6 g/day	Diarrhea (grade 1)	(+)
2	7.2 g/day	Skin eruption (grade 2)	(+)
3	7.2 g/day	Anorexia (grade 1)	(−)
		Fatigue (grade 1)	
4	7.2 g/day	Chills (grade 1)	(−)
		Constipation (grade 1)	

pared with patients with HCV serotype 1, but the HCV serotype had no significant influence on the virological response to bLF treatment.

Toxicity All 45 patients received at least one dose of bLF and were therefore eligible for toxicity assessment. The bLF treatment was generally well tolerated, and no patient showed any serious adverse event. Minor, transient treatment-related symptoms occurred in four patients, and these adverse effects appeared to be dose-dependent; three of the four patients received bLF of 7.2 g/day, while no symptoms occurred in patients receiving bLF of 1.8 g/day (Table IV). Therapy was continued without any specific treatment in two of the four patients, but the doses were reduced in the remaining two. Moreover, there were no clinically significant abnormalities in laboratory values during or after treatment.

DISCUSSION

The incidence of new HCV infections has markedly declined in recent years because of the availability of HCV testing of donated blood.[27] However, the number of patients infected with HCV remains high, and the number of CH-C patients likely to develop clinically significant liver cirrhosis has not yet peaked. CH-C may have a negligible clinical impact over the first 1 to 2 decades in the majority of patients, but without a highly effective treatment, patients with CH-C usually suffer from liver cirrhosis and eventually HCC.[2,3] The primary goal in the treatment of CH-C is to achieve a complete resolution of CH (at least a decrease in progression to cirrhosis) and an inhibition of HCC development. However, current therapies for CH-C, including IFN, are less than optimal even with new approaches such as pegylated IFN and IFN/ribavirin combination.[6–10] Furthermore, the adverse effects of IFN therapy may significantly impair the quality of life of patients during the course of treatment. Therefore, there remains a critical need for newer and more effective treatments without severe adverse effects for CH-C.

LF has several biological activities, which include antiviral activity and immunomodulatory functions such as natural killer cell activation.[11,12] Anti-viral activity of LF has been reported for some viruses, and LF is the first physiological substance other than IFN found to show anti-HCV activity.[13–19] With respect to mechanisms of anti-viral activity of LF, two distinct mechanisms were reported; the direct interaction of LF with the cells and the interaction of LF with the virus. In the case of HCV, the anti-viral activity *in vitro* is due to the direct binding of LF to HCV; the interaction between LF and HCV occurred immediately after mixing LF and serum containing HCV, resulting in the inhibition of adsorption and/or internalization of the HCV-LF complex into human hepatocytes.[17–19] In contrast, LF showed no anti-HCV activity after adsorption and/or internalization of HCV into human hepatocytes.

In this dose-response trial, we examine the relation between the dose of bLF and the effect of bLF on serum HCV RNA and ALT levels in patients with CH-C. The patients who entered at each of the three dose levels (bLF of 1.8, 3.6, and 7.2 g/day) received an 8-week course of bLF and then were followed for the next 8 weeks. There was no significant relationship between the dose of bLF and the effect of bLF on serum ALT or HCV RNA levels. However, a biochemical (a 50% or greater decrease in serum ALT level) and/or virological (a 50% or greater decrease in HCV RNA level) response occurred in some CH-C patients; the durations of the biochemical responses were 5 and 6 months, and the durations of the virological responses ranged from 6 to 10 months. There was a major discrepancy between the durations of the responses in the present trial and the pilot study; in the pilot study, two of the three responders relapsed 2 weeks after cessation of the treatment.[20] Although the reason for the conflicting results remains unclear, future trials using a large number of patients may resolve this issue.

With respect to toxicity, although mild, transient treatment-related symptoms, which appeared to be dose-dependent, occurred infrequently during bLF treatment, no clinically significant abnormalities were observed in laboratory values. Many patients with chronic liver disease, particularly cirrhosis, have leukocytopenia and thrombocytopenia, which limit the dose and duration of IFN therapy. Therefore, bLF treatment appears advantageous in comparison with IFN in terms of toxicity. Based on these results regarding the efficacy and toxicity, we suggest that the recommended dose for future trials using this schedule is 1.8 g/day.

Serum HCV RNA levels decreased even after cessation of bLF treatment in some patients; while the virological response was observed in only four patients at the end of treatment, eight patients achieved a virological response at week 8 after the end of treatment. These virological responses were highly correlated with a decrease in the serum ALT levels. Since progression to cirrhosis in patients with CH-C depends on the severity of hepatic

inflammation, bLF treatment, by reducing hepatic inflammation, may prevent progression of CH even in the presence of persistent viremia. Age was associated with the virological response to bLF treatment, but serum HCV RNA levels, HCV serotype, or hepatic fibrosis had no significant influence on the response to bLF treatment. Accordingly, the beneficial effect of bLF treatment may extend to subgroups of patients in whom IFN therapy has historically been unsuccessful, such as patients with high HCV RNA levels, HCV serotype 1, or advanced fibrosis.

LF is present in the secondary granules of neutrophils, and plasma LF is predominantly granulocyte-derived.[28] Activated granulocytes release LF into circulation, and a rise in plasma LF concentrations is observed in some situations such as inflammation and pregnancy.[29,30] Therefore, granulocytes may be associated with the response to bLF treatment. However, in the present trial, the virological response was not influenced by baseline granulocyte count, although plasma LF concentrations may or may not correlate with the granulocyte count, depending on the magnitude of degranulation and perhaps the contribution of other organs to the plasma content of LF.[30–32]

Despite the potential anti-HCV activity of bLF shown in the current trial, several questions remain concerning bLF in the treatment of CH-C. It is unclear if bLF really has anti-HCV activity in patients with CH-C, or what the optimal treatment duration is for CH-C. It is also necessary to clarify the criteria for selecting patients to be treated with bLF, and whether a response to bLF will alter the natural history of CH-C, reducing the likelihood of future development of cirrhosis, end-stage liver disease, HCC, or death. It must also be clarified if bLF has substantial anti-HCV activity in CH-C patients who showed no response to previous treatment with IFN, and in CH-C patients with relapse after IFN therapy. In addition, it needs to be clarified if the combination therapy of bLF and IFN would be a more effective means of clinical treatment. To resolve these questions and to define the exact role of bLF treatment in the treatment of CH-C, trials using large numbers of patients with a longer observation period are mandatory. We are currently conducting a double-blind randomized controlled trial comparing bLF with placebo to clarify the anti-HCV activity of bLF in patients with CH-C.

ACKNOWLEDGMENTS

This study was sponsored by Morinaga Milk Industry Co. We wish to thank Ms. Keiko Kondo for help with data management and manuscript preparation. Special thanks are due to Dr. Hiroyuki Tsuda (National Cancer Center Research Institute), Dr. Nobuyuki Kato (Okayama University Medical School), and Dr. Akinori Nozawa (Yokohama City University) as scientific contributors.

(Received April 22, 2002/Revised June 10, 2002/Accepted June 19, 2002)

REFERENCES

1) Di Bisceglie, A. M. Hepatitis C and hepatocellular carcinoma. *Hepatology*, **26** (Suppl. 1), 34S–38S (1997).
2) Takano, S., Yokosuka, O., Imazeki, F., Tagawa, M. and Omata, M. Incidence of hepatocellular carcinoma in chronic hepatitis B and C: a prospective study of 251 patients. *Hepatology*, **21**, 650–655 (1995).
3) Niederau, C., Lange, S., Heintges, T., Erhardt, A., Buschikamp, M., Hurter, D., Nawrocki, M., Kruska, L., Hensel, F., Petry, W. and Haussingger, D. Prognosis of chronic hepatitis C: results of a large, prospective cohort study. *Hepatology*, **28**, 1687–1695 (1998).
4) Hoofnagle, J. H. and Di Bisceglie, A. M. The treatment of chronic viral hepatitis. *N. Engl. J. Med.*, **336**, 347–356 (1997).
5) Davis, G. L. Current therapy for chronic hepatitis C. *Gastroenterology*, **118**, S104–S114 (2000).
6) McHutchison, J. G., Gordon, S. C., Schiff, E. R., Shiffman, M. L., Lee, W. M., Rustgi, V. K., Goodman, Z. D., Ling, M. H., Cort, S. and Albrecht J. K. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. *N. Engl. J. Med.*, **339**, 1485–1492 (1998).
7) Davis, G. L., Esteban-Mur, R., Rustgi, V., Hoefs, J., Gordon, S. C., Trepo, C., Shiffman, M. L., Zeuzem, S., Craxi, A., Ling, M. H. and Albrecht, J. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. *N. Engl. J. Med.*, **339**, 1493–1499 (1998).
8) Poynard, T., Marcellin, P., Lee, S. S., Niederau, C., Minuk, G. S., Ideo, G., Bain, V., Heathcote, J., Zeuzem, S., Trepo, C. and Albrecht, J. Randomised trial of interferon α2b plus ribavirin for 48 weeks or for 24 weeks versus interferon α2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. *Lancet*, **352**, 1426–1432 (1998).
9) Zeuzem, S., Feinman, S. V., Rasenack, J., Heathcote, E. J., Lai, M.-Y., Gane, E., O'Grady, J., Reichen, J., Diago, M., Lin, A., Hoffman, J. and Brunda, M. J. Peginterferon alfa-2a in patients with chronic hepatitis C. *N. Engl. J. Med.*, **343**, 1666–1672 (2000).
10) Heathcote, E. J., Shiffman, M. L., Cooksley, G. E., Dusheiko, G. M., Lee, S. S., Balart, L., Reindollar, R., Reddy, R. K., Wright, T. L., Lin, A., Hoffman, J. and De Pamphilis, J. Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis. *N. Engl. J. Med.*, **343**, 1673–1680 (2000).
11) Lonnerdal, B. and Iyer, S. Lactoferrin: molecular structure and biological function. *Annu. Rev. Nutr.*, **15**, 93–110

(1995).
12) Levay, P. F. and Viljoen, M. Lactoferrin: a general review. *Haematologica*, **80**, 252–267 (1995).
13) Hasegawa, K., Motsuchi, W., Tanaka, S. and Dosako, S. Inhibition with lactoferrin of *in vitro* infection with human herpes virus. *Jpn. J. Med. Sci. Biol.*, **47**, 73–85 (1994).
14) Fujihara, T. and Hayashi, K. Lactoferrin inhibits herpes simplex virus type-1 (HSV-1) infection to mouse cornea. *Arch. Virol.*, **140**, 1469–1472 (1995).
15) Defer, M. C., Dugas, B., Picard, O. and Damais, C. Impairment of circulating lactoferrin in HIV-1 infection. *Cell. Mol. Biol.*, **41**, 417–421 (1995).
16) Harmsen, M. C., Swart, P. J., de Bethune, M. P., Pauwels, R., De Clercq, E., The, T. H. and Meijer, D. K. Antiviral effects of plasma and milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication *in vitro*. *J. Infect. Dis.*, **172**, 380–388 (1995).
17) Ikeda, M., Sugiyama, K., Tanaka, T., Tanaka, K., Sekihara, H., Shimotohno, K. and Kato, N. Lactoferrin markedly inhibits hepatitis C virus infection in cultured human hepatocytes. *Biochem. Biophys. Res. Commun.*, **245**, 549–553 (1998).
18) Ikeda, M., Nozaki, A., Sugiyama, K., Tanaka, T., Naganuma, A., Tanaka, K., Sekihara, H., Shimotohno, K., Saito, M. and Kato, N. Characterization of antiviral activity of lactoferrin against hepatitis C virus infection in human cultured cells. *Virus Res.*, **66**, 51–63 (2000).
19) Yi, M., Kaneko, S., Yu, D. Y. and Murakami, S. Hepatitis C virus envelope proteins bind lactoferrin. *J. Virol.*, **71**, 5997–6002 (1997).
20) Tanaka, K., Ikeda, M., Nozaki, A., Kato, N., Tsuda, H., Saito, S. and Sekihara, H. Lactoferrin inhibits hepatitis C virus viremia in patients with chronic hepatitis C: a pilot study. *Jpn. J. Cancer Res.*, **90**, 367–371 (1999).
21) Japan Society for Cancer Therapy. Criteria for the evaluation of the clinical effects of solid cancer chemotherapy. *J. Jpn. Soc. Cancer Ther.*, **28**, 101–130 (1993).
22) Shibuya, M. Adverse drug reaction criteria of the Japan Society for Cancer Therapy. *Jpn. J. Cancer Chemother.*, **24**, 2036–2041 (1997).
23) Tsukiyama-Kohara, K., Kohara, M., Yamaguchi, K., Maki, N., Toyoshima, A., Miki, K., Tanaka, S., Hattori, N. and Nomoto, A. A second group of hepatitis C viruses. *Virus Genes*, **5**, 243–254 (1991).
24) Tanaka, T., Tsukiyama-Kohara, K., Yamaguchi, K., Yagi, S., Tanaka, S., Hasegawa, A., Ohta, Y., Hattori, N. and Kohara, M. Significance of specific antibody assay for genotyping of hepatitis C virus. *Hepatology*, **19**, 1347–1353 (1994).
25) Simmonds, P., McOmish, F., Yap, P. L., Chan, S.-W., Lin, C. K., Dusheiko, G., Saeed, A. A. and Holmes, E. C. Sequence variability in the 5′ non-coding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity. *J. Gen. Virol.*, **74**, 661–668 (1993).
26) Bedossa, P., Poynard, T. and for The METAVIR Cooperative Study Group. An algorithm for the grading of activity in chronic hepatitis C. *Hepatology*, **24**, 289–293 (1996).
27) Armstrong, G. L., Alter, M. J., McQuillan, G. M. and Margolis, H. S. The past incidence of hepatitis C virus infection: implications for the future burden of chronic liver disease in the United States. *Hepatology*, **31**, 777–782 (2000).
28) Iyer, S. and Lonnerdal, B. Lactoferrin, lactoferrin receptors and iron metabolism. *Eur. J. Clin. Nutr.*, **47**, 232–241 (1993).
29) Sykes, J. A., Thomas, M. J., Goldie, D. J. and Turner, G. M. Plasma lactoferrin levels in pregnancy and cystic fibrosis. *Clin. Chim. Acta*, **122**, 385–393 (1982).
30) Baynes, R., Bezwoda, W., Bothwell, T., Khan, Q. and Mansoor, N. The non-immune inflammatory response: serial changes in plasma iron, iron-binding capacity, lactoferrin, ferritin and C-reactive protein. *Scand. J. Clin. Lab. Invest.*, **46**, 695–704 (1986).
31) Hansen, N. E., Malmquist, J. and Thorell, J. Plasma myeloperoxidase and lactoferrin measured by radioimmunoassay: relations to neutrophil kinetics. *Acta Med. Scand.*, **198**, 437–443 (1975).
32) Olofsson, T., Olsson, I., Venge, P. and Elgefors, B. Serum myeloperoxidase and lactoferrin in neutropenia. *Scand. J. Haematol.*, **18**, 73–80 (1977).

STEM CELLS®
Original Article

The Role of PGE_2 in the Differentiation of Dendritic Cells: How Do Dendritic Cells Influence T-Cell Polarization and Chemokine Receptor Expression?

JE-JUNG LEE,[a,b] MASAO TAKEI,[c] SHINICHI HORI,[b] YOSHIKO INOUE,[d] YUKIE HARADA,[b] RYUJI TANOSAKI,[b] YOSHINOBU KANDA,[b] MASAYUKI KAMI,[b] ATSUSHI MAKIMOTO,[b] SHIN MINEISHI,[b] HIROYUKI KAWAI,[c] AKIHIRO SHIMOSAKA,[c] YUJI HEIKE,[d] YOSHINORI IKARASHI,[d] HIRO WAKASUGI,[d] YOICHI TAKAUE,[b] TAI-JU HWANG,[e] HYEOUNG-JOON KIM,[a] TADAO KAKIZOE[f]

[a]Department of Internal Medicine, Chonnam National University Medical School, Gwangju, Republic of Korea; [b]Division of Transplantation/Immunotherapy, National Cancer Center Hospital, Tokyo, Japan; [c]Kirin Brewery Company, Tokyo, Japan; [d]Pharmacology Division, Research Institute, National Cancer Center, Tokyo, Japan; [e]Department of Pediatrics, Chonnam National University Medical School, Gwangju, Republic of Korea; [f]National Cancer Center Hospital, Tokyo, Japan

Key Words. *Blood · Dendritic Cells · Th1/Th2 · Cell trafficking*

ABSTRACT

The role of prostaglandin E_2 (PGE_2) in the function of dendritic cells (DCs), T-cell polarization, and expression of chemokine receptors was evaluated in human cells. Immature DCs were generated from peripheral blood $CD14^+$ cells using a combination of GM-CSF and interleukin-4 (IL-4) with or without PGE_2. On day 6, maturation of DCs was induced by the addition of tumor necrosis factor alpha with or without PGE_2. DCs harvested on day 6 (immature DCs) or day 9 (mature DCs) were examined using functional assays. In the presence of PGE_2, immature and mature DCs showed, phenotypically, a lower expression of CD1a and, functionally, a higher allostimulatory capacity at a high DC/T-cell ratio than control cells cultured in the absence of PGE_2. DCs cultured in the presence of PGE_2 induced the differentiation of naïve T cells toward a helper T-cell type 1 (Th1) response, which was independent of IL-12 secretion in the basal state despite a slightly lower interferon gamma secretion compared with control cells. However, the function of cytotoxicity-stimulating autologous T cells was not augmented by the addition of PGE_2. Immature DCs expressed the inflammatory chemokine receptors, CCR1 and CXCR4, but not CCR6, regardless of the presence or absence of PGE_2. Mature DCs expressed CCR7 equally, measured using a migration test and the measurement of calcium flux with macrophage inflammatory protein-3β and reverse transcription-polymerase chain reaction assay in all of the groups. All of these findings suggest that PGE_2 affects the DC-promoted differentiation of naïve T cells to a Th1 response in the basal state, without affecting chemokine receptor expression on DCs. *Stem Cells 2002;20:448-459*

Correspondence: Yoichi Takaue, M.D., Hematopoietic Stem Cell Transplant Unit, National Cancer Center Hospital, 1-1 Tsukiji 5-Chome, Chuo-ku, Tokyo 104-0045, Japan. Telephone: 81-3-3542-2511; Fax: 81-3-3542-3815; e-mail: ytakaue@ncc.go.jp *Received April 25, 2002; accepted for publication June 26, 2002.*

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells for initiating cellular immune responses through the stimulation of naïve T cells, and their action is mainly due to the constitutive and upregulated expression of molecules related to adhesion, major histocompatibility complex (MHC), and a costimulatory pathway [1-3]. Previously, the concept of a predetermined "one cell type-one type of response," e.g., myeloid DCs induce helper T-cell type 1 (Th1) responses while lymphoid/plasmacytoid DCs induce Th2 responses, was dominant [4]. However, many investigators have since reported that DCs promote T-cell polarization depending on environmental instructions in the culture conditions or on activation signals, rather than an intrinsic ontogeny [5, 6]. DCs that generate Th1 responses may be used in clinically applicable therapeutic modalities for pathologic conditions that are caused by infections or malignant disorders, by secreting interleukin-2 (IL-2) and interferon gamma (IFN-γ) to facilitate T-cell-mediated cytotoxicity [7-9]. In contrast, DCs that generate Th2 responses may be clinically used in conditions in which Th1 responses are disturbed, e.g., transplantation, contact allergy, or autoimmune disorders, by secreting cytokines, including IL-4, IL-5, IL-6, and IL-10, to help B cells secrete protective antibodies [8, 10].

Prostaglandin E_2 (PGE_2) is produced by stromal cells and tissue-infiltrating mononuclear cells, and is an important inflammatory mediator that elevates intracellular cyclic AMP (cAMP). PGE_2 inhibits IL-2 and IFN-γ production by Th1-type cells, but not the production of IL-4 by Th2-type cells [11-13], and thereby, regulates the differentiation of naïve $CD4^+$ T cells into Th2-type $CD4^+$ T cells in vivo [11-13]. However, somewhat contradictory results have been reported regarding T-cell polarization induced by DCs that are cultured in the presence of PGE_2. *Kalinski* and coworkers [6, 14-16] reported that immature and mature DCs, generated in the presence of PGE_2, either initially or during terminal maturation, induced naïve T cells toward a Th2 response by impairing the production of IL-12. On the other hand, other investigators reported the induction of a Th1 response mediated by the greater production of IL-2 and IFN-γ with the addition of PGE_2, which resulted in higher IL-12 production [17-19]. Thus, the definitive effect of PGE_2 in the differentiation of DCs remains to be established.

Chemokines play an important role in trafficking DCs to lymphoid organs [20]. Immature DCs express various inflammatory chemokine receptors, such as CCR1, CCR4, CCR5, CCR6, and CXCR4. As maturation proceeds, DCs upregulate the constitutive expression of chemokine receptor CCR7 and downregulate inflammatory chemokine receptors, which permits DCs to be localized in lymphoid tissues where pulsed DCs stimulate effector T cells via the interaction with T-cell receptors [20-22]. In addition, previous reports have suggested that the conditions used to culture DCs affect the expression of chemokine receptors [23, 24]. Thus, this field appears to be complex, and factors that affect DC function through the expression of chemokine receptors or interaction with their ligands need to be defined.

Hence, in this study, we investigated the roles of PGE_2 in the regulation of DC function, particularly focusing on the polarization of T cells and the modification of the expression of chemokine receptors.

Materials and Methods

Medium, Reagents, and Monoclonal Antibodies

The medium used was RPMI-1640 (GIBCO-BRL; Grand Island, NY; http://www.invitrogen.com) supplemented with 10% fetal calf serum (FCS; Hyclone; Logan, UT; http://www.hyclone.com) and 1% penicillin-streptomycin (GIBCO-BRL). Recombinant human GM-CSF was provided by Kirin Brewery Company (Tokyo, Japan; http://www1.kirin.co.jp/english/r_d/pha/index.html). Recombinant IL-4, tumor necrosis factor alpha (TNF-α), and IL-2 were purchased from R&D systems (Minneapolis, MN; http://www.rndsystems.com). PGE_2 was purchased from ICN Biomedicals (Aurora, OH; http://www.icnbiomed.com) and rMIP-3β/ELC was from Dako (Kyoto, Japan; http://www.dako.dk).

Fluorescence-activated cell sorting (FACS) analysis was performed using mouse monoclonal antibodies (mAbs) against fluorescein isothiocyanate-labeled CD14 (CD14-FITC), HLA-DR-FITC, IFN-γ-FITC, phycoerythrin-labeled CD80 (CD80-PE), CD54-PE, IL-4-PE, and CD4-Percp, which were purchased from Becton Dickinson (San Jose, CA; http://www.bd.com). CD40-FITC and CD86-FITC antibodies were from PharMingen (San Diego, CA; http://www.bdbiosciences.com/pharmingen), and mAbs against CD45RA-FITC, CD1a-PE, CD45RO-PE, and CD83-PE were from Coulter-Immunotech (Miami, FL; http://www.coulter.com). Biotinylated mAbs against CCR1, CXCR4, and CCR6 were purchased from Dako. Isotype controls were run in parallel. Cell debris was eliminated from the analysis by forward and side scatter gating. The samples were acquired on a FACS Caliber cell sorter (Becton Dickinson) and analyzed with CellQuest software (Becton Dickinson).

Generation of Immature DCs and Induction of Mature DCs

$CD14^+$ cells isolated from leftover peripheral blood stem cell (PBSC) products, which were collected from healthy donors mobilized with G-CSF for allogeneic trans-

plantation after obtaining a consent form, were used to generate DCs. In brief, the PBSC products produced 2-6 × 10^8 cells after washing the material remaining in the apheresis bags. $CD14^+$ cells were isolated from the PBSC products (>85% purity) using the magnetic activated cell sorter (MACS) according to the manufacturer's instructions (Miltenyi Biotec; Auburn, CA; http://www.miltenyi biotec.com). To generate immature DCs, the $CD14^+$ cells were cultured at a density of 1 × 10^6 cells per 1 ml of culture medium with one of the following combinations of cytokines: A) GM-CSF (50 ng/ml) and IL-4 (50 ng/ml) or B) GM-CSF (50 ng/ml), IL-4 (50 ng/ml), and PGE_2 (10^{-7} M). Freshly prepared cytokines were added every other day. Maturation of DCs was induced by adding TNF-α (50 ng/ml) with or without PGE_2 (10^{-7} M) to each group of cultures on day 6. DCs were harvested as immature (day 6) or mature (day 9) cells for morphologic, phenotypic, or functional evaluations. Additionally, the culture supernatants were collected on the same days and frozen at –20°C until measurement of IL-10 and IL-12 levels using enzyme-linked immunosorbent assays (ELISA).

Immature or mature DCs (3 × 10^4 cells) were then prepared on cytospin slides with centrifugation at 400 revolutions per minute (rpm) for 5 minutes. These slides were dried, fixed with Carnoy's solution (ethanol:acetic acid:chloroform = 6:1:3), stained with May-Grunwald-Giemsa stain, and visualized by light microscopy.

T-Cell Preparation

$CD3^+$ cells for the mixed lymphocyte reaction (MLR) assay were obtained from the peripheral blood of a healthy donor using human T-cell enrichment columns (R&D Systems). Naive $CD4^+CD45RA^+$ T cells for the cytokine assay were isolated from the PBSC products by two-step positive selection using MACS. $CD8^+$ cells for the cytotoxic assay were also isolated from autologous PBSC products by positive selection using MACS. The purity of isolated cells was >90% for $CD3^+$ cells and >95% for naïve $CD4^+CD45RA^+$ T cells or $CD8^+$ cells.

Endocytic Activity

Cells (1 × 10^5), suspended in 100 μl of culture medium, were incubated with FITC-dextran (20 μg/ml) for 1 hour at 37°C in a water bath or at 4°C on ice, and then washed thoroughly with cold buffer consisting of phosphate-buffered saline (PBS), 5 mM EDTA, and 2% FCS. The samples were acquired on a FACS Caliber cell sorter, with 10^4 gating events for each sample, and analyzed with CellQuest software. The results were expressed as a mean fluorescence intensity (MFI) index calculated as the ratio of the MFI of the sample to the MFI of an isotype-matched control.

Allogeneic MLR

Allogeneic $CD3^+$ cells (5 × 10^4/well), obtained from healthy donor blood, were cocultured in 96-well, round-bottomed culture plates with graded doses (2 × 10^2 – 5 × 10^4) of irradiated (30 Gy) DCs that were harvested from each group on day 6 or 9 of culture. After 5 days, the cells were pulsed with 1 Ci [^{3}H]-methylthymidine per well for 16 hours, harvested, and analyzed in a liquid scintillation counter. The results were expressed as the mean counts per minute (cpm) ± the standard error (SE) of results obtained with four different samples, each performed in triplicate.

Intracellular Cytokine Staining

DCs (1.05 × 10^5 cells/500 μl) that were harvested on day 6 or 9 were primed with 50 ng/ml staphylococcal exotoxins (Toxin Technology Inc.; Sarasota, FL; http://www.toxintechnology.com), which contain an enterotoxin (SEA-SEE), exfoliative toxins (ETA and ETB), and toxic shock syndrome toxin (TSST-1), for 1 hour at 37°C and then irradiated (30 Gy). DCs were cocultured with naïve $CD4^+CD45RA^+$ T cells (5 × 10^5/well) in the culture medium at ratios of 1:5 (total volume of 600 μl) and 1:300 (total volume of 500 μl) in 24-well plates. On day 5, the cells were washed out completely and expanded with fresh medium containing 500 U/ml of IL-2. Two hundred fifty microliters of culture supernatant were then replaced with medium of the same concentration every 3 days. On day 14, the supernatant was collected and frozen at –20°C until ELISA assays for IFN-γ and IL-4 were performed. The intracellular cytokine concentrations of the harvested T cells were measured by FACS analysis, as described previously. Briefly, T cells (10^6) were stimulated with phorbol myristate acetate (PMA) (10 ng/ml) and ionomycin (1 μg/ml) for 4 hours in a 37°C water bath. Brefeldin A (10 μg/ml) was added during the last 2 hours of incubation to prevent cytokine secretion. Cells were collected, fixed with 1% paraformaldehyde (Becton Dickinson), permeabilized with a commercial solution (Becton Dickinson), and stained with FITC-labeled anti-IFN-γ (IgG2a) and PE-labeled anti-IL-4 (IgG1) mAbs. The samples were processed by a FACS Caliber cell sorter, with at least 10^4 gating events for each sample, and analyzed with CellQuest software.

Cytotoxic T-Lymphocyte (CTL) Assay

Mature DCs (5 × 10^5) cultured from $HLA\text{-}A24^+$ donors were loaded with 10 μM Epstein-Barr virus (EBV)-derived peptide (TYGPVFMCL), which can bind to HLA-A2402 as reported by *Lee et al.* [25], for 2 hours at 37°C. In 24-well plates, autologous $CD8^+$ T cells were cocultured as effector cells at a ratio of 2:1 with DCs in 2 ml of medium (RPMI 1640:AIM-V = 1:1) containing 10% FCS (Sigma; St. Louis, MO; http://www.sigmaaldrich.com), 1% penicillin-strepto-

mycin (GIBCO-BRL), and 0.1 mM/l nonessential amino acids supplemented with 100 U/ml IL-2 for 10 days; half the medium was changed every 3 days. BEC-2 cell lines (HLA-A2402) and Bamb-2 cell lines (HLA-A1/A26) generated by EBV-transformed B-lymphoblastoid cell line from an EBV^+ healthy donor were kindly provided by *Dr. Itoh* (Kurume University School of Medicine; Kurume, Japan). The BEC-2 cells and Bamb-2 cells, as target cells, were loaded at a concentration of 1×10^6 cells/ml with 10 μM EBV peptide and incubated for 2 hours at 37°C in 5% CO_2. Effector cells were cocultured with target cells at effector-to-target ratios of 2:1, 5:1, and 10:1 in 96-well round-bottomed culture plates (total volume of 200 μl) in duplicate or triplicate. After overnight incubation at 37°C in 5% CO_2, the human IFN-γ concentration of the supernatant was measured (pg/ml) using ELISA according to the manufacturer's instructions (OptEIA™ Human IFN-γ Set; PharMingen).

Cytokine Assay by ELISA

IL-10 (limit of sensitivity, 3.9 pg/ml) and IL-12 (limit of sensitivity, 0.5 pg/ml) were measured in the supernatant collected at day 6 or 9 of DC culture using Quantikine Immunoassay Kits (R&D Systems) according to the manufacturer's instructions. IL-4 (limit of sensitivity, 0.13 pg/ml) and IFN-γ were measured in the supernatant of naïve $CD4^+CD45RA^+$ cells obtained at day 14. Cytokines were quantified using a microplate reader at 450-490 nM, plotted against a standard curve with the cytokines, and expressed in pg/ml.

Transmigration Assay

To evaluate the chemotactic effects of DCs stimulated with chemokines, a double-chamber system was used, with previously described modifications [26]. Briefly, polycarbonate membranes with 8-μm pore size filters (Chemotaxicell™; Kurabo; Osaka, Japan; http://www.kurabo.co.jp) were placed on 24-well culture plates to separate the upper and lower chambers. Macrophage inflammatory protein (MIP)-3β for mature DCs was seeded at concentrations of 1, 10, and 100 nM diluted with 500 μl of culture medium in the lower chambers. Next, $0.5\text{-}1 \times 10^5/100$ μl mature DCs were added to the upper chambers. After overnight incubation at 37°C in 5% CO_2, the cells that migrated into the lower chambers were collected. Using a Coulter counter, only cells larger than 12 μm were counted, to eliminate lymphocyte contamination. The results were expressed as a net migration percentage calculated as: (number of cells that migrated into the lower chamber containing chemokines—number of cells that migrated in medium alone)/total number of cells loaded in the upper chamber × 100.

Calcium Flux Measurement

Mature DCs, at a concentration of 1×10^6 cells/ml, were loaded with 5 μg/ml Fluo-3AM (Calbiochem; San Diego, CA), incubated for 30 minutes in a 37°C water bath, and centrifuged for 10 minutes at 1,500 rpm. The DCs were then resuspended in RPMI with 5% FCS at 1×10^6 cells/ml. Calcium flux was measured at 450 nM as a function of time in response to 100 nM MIP-3β for mature DCs, with 3 mM EGTA and as a negative control and ionomycin (10 ng/ml) as a positive control, using a FACS Caliber cell sorter, and analyzed using FlowJo software (Becton Dickinson).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for CCR7

Total RNA was extracted from $1\text{-}2 \times 10^6$ mature DCs using an RNeasy Mini Kit (Qiagen; Hilden, Germany; http://www.qiagen.com). The cDNA synthesized from total RNA by Moloney murine leukemia virus reverse transcriptase (Stratagene; Austin, TX; http://www.stratagene.com) was subjected to RT-PCR using 30 cycles at 94°C for 0.5 minutes, 57.5°C for 1 minute, and 72°C for 1 minute for CCR7 (TaKaRa; Tokyo, Japan; http://www.takara bio.co.jp/english/index.htm). The sense and anti-sense oligonucleotide primers for CCR7 were 5′-CGCGTC-CTTCTCATCAGCAA-3′ and 5′-GTCCCGACAGGAA-GACCACT-3′, respectively. PCR products were identified by electrophoresis on 2% agarose gels that were photographed.

Statistical Analysis

Values are presented as the mean ± SE. The Mann-Whitney *U* test was used to compare values between subgroups using SPSS 10.0 software. p values <0.05 were considered statistically significant.

Results

Morphology, Phenotypic Features, and Endocytic Activity

With May-Grünwald-Giemsa staining, cells cultured with or without PGE_2 for 6 or 9 days showed similar typical features of immature DCs (large cells with an irregular outline and a few veils) or mature DCs (large, veiled, nonadherent appearance and highly motile). However, when the cultured cells on the plates were directly observed without staining under phase-contrast microscopy on day 9, the cells cultured in the presence of PGE_2 had less branching and fewer veiled structures than those without PGE_2 (data not shown).

With FACS analysis, the expression of CD1a, CD40, and CD83 in immature DCs treated with PGE_2 was significantly lower than that in control cells without PGE_2 (means 8%, 40%, and 2% versus 73%, 86%, and 11%, respectively;

Table 1. Comparison of phenotypes of immature DCs (iDC) cultured with GM-CSF + IL-4 and immature DCs (PGE_2-iDC) cultured in the presence of PGE_2 on day 6

	iDC	PGE_2-iDC	*p* value
CD14	12.3 ± 3.5	85.4 ± 3.4	0.001
CD1a	72.9 ± 6.8	8.1 ± 1.9	0.001
HLA-DR	98.9 ± 0.6	98.9 ± 0.5	0.953
CD80	57.2 ± 8.4	40.6 ± 14.3	0.298
CD83	11.3 ± 3.2	2.3 ± 0.8	0.028
CD86	52.7 ± 6.2	69.8 ± 6.6	0.105
CD40	86.4 ± 6.7	40.0 ± 9.0	0.004
CD54	99.0 ± 0.2	97.3 ± 1.5	0.245

Values are percentages ± SE.

$p = 0.001$, $p = 0.004$, and $p = 0.028$, respectively) as shown in Table 1. The expression of CD14 on immature DCs was retained in cells cultured with PGE_2, while this was lost in control cells (mean 86% versus 12%, $p = 0.001$, Table 1). Other phenotypes, including HLA-DR, CD80, CD86, and CD54 were similarly highly expressed on both cells, regardless of the presence of PGE_2.

When mature DCs, which were treated with TNF-α alone as a maturating agent, were evaluated by FACS, the cells with added PGE_2 showed a significantly lower expression of CD1a than control cells (88% versus 65%, $p = 0.024$, Table 2). TNF-α also induced a similar maturation pattern in immature DCs that were initially cultured with PGE_2. CD1a expression in these cells was almost completely recovered when PGE_2 was removed compared with the continuous presence of PGE_2 (78% versus 19%, $p = 0.004$, Table 2). However, the DCs from which PGE_2 had been removed showed a lower expression of CD83 and CD86, compared with those in the continuous presence of PGE_2 (means of 62% and 72% versus 88% and 92%, respectively, $p = 0.016$ and $p = 0.01$, Table 2).

We then used FITC-dextran to measure mannose receptor-mediated endocytosis in immature and mature DCs induced by various cytokine combinations. In immature DCs, the MFI index tended to be higher in the presence of PGE_2 than in the controls (29 ± 11 versus 16 ± 6). However, the ability to take up soluble antigen decreased as DCs matured (data not shown).

DC with Added PGE_2 Showed a High MLR at a High DC/T-Cell Ratio

Immature DCs cultured with PGE_2 had a higher capacity to stimulate allogeneic $CD3^+$ T cells than control cells at a higher DC/T-cell ratio ($p = 0.003$ and $p = 0.007$ at ratios of 1:1 and 1:2, respectively, Fig. 1A). On the other hand, there were lower allostimulatory effects in the MLR at a lower cell ratio, although variations were noted among different samples ($p < 0.001$ and $p = 0.0013$ at ratios of 1:16 and 1:128, respectively, Fig. 1A). These findings suggest that PGE_2 affected the allostimulatory capacity of DCs depending upon the DC/T-cell ratio. The allostimulatory capacity of mature DCs on $CD3^+$ T cells was also higher when the cells were cultured with PGE_2 than without PGE_2 at a high cell-dose ratio (Fig. 1B).

Effects of PGE_2 on DC-Mediated Th1 Polarization of Naive T Cells

When immature DCs, which had been cultured in the presence of PGE_2, were used to induce the differentiation of naïve T cells, there was a shift toward Th1 polarization, as judged by elevated IFN-γ levels, minimal expression of IL-4 by intracellular cytokine staining, and ELISA assays, although a slightly lower excretion of IFN-γ than in controls was observed in the presence of PGE_2 at high DC/T-cell ratios (1:5). Mature DCs also showed a higher level of IFN-γ and a lower level of IL-4, but there was no difference in cytokine levels between different culture conditions

Table 2. Comparison of phenotypes of mature DCs cultured with various cytokine combinations on day 9

	iDC + TNF-α	iDC + TNF-α + PGE_2	PGE_2-iDC + TNF-α	PGE_2-iDC + TNF-α + PGE_2
CD14	0.6 ± 0.2	1.0 ± 0.2	5.0 ± 0.9	5.7 ± 1.4
CD1a	87.9 ± 2.6	65.1 ± 8.4	77.8 ± 6.5	18.9 ± 5.4
HLA-DR	99.8 ± 0.0	99.7 ± 0.1	99.7 ± 0.1	99.5 ± 0.2
CD80	95.4 ± 2.7	96.3 ± 1.7	94.9 ± 3.1	96.1 ± 1.6
CD83	85.9 ± 2.4	90.0 ± 3.1	61.8 ± 7.4	87.9 ± 3.7
CD86	93.6 ± 1.6	97.5 ± 0.6	72.1 ± 6.3	92.3 ± 3.9
CD40	99.4 ± 0.3	96.8 ± 1.4	99.6 ± 0.3	99.2 ± 0.4
CD54	98.5 ± 0.7	95.2 ± 1.9	99.7 ± 0.1	99.4 ± 0.3

Values are percentages ± SE.

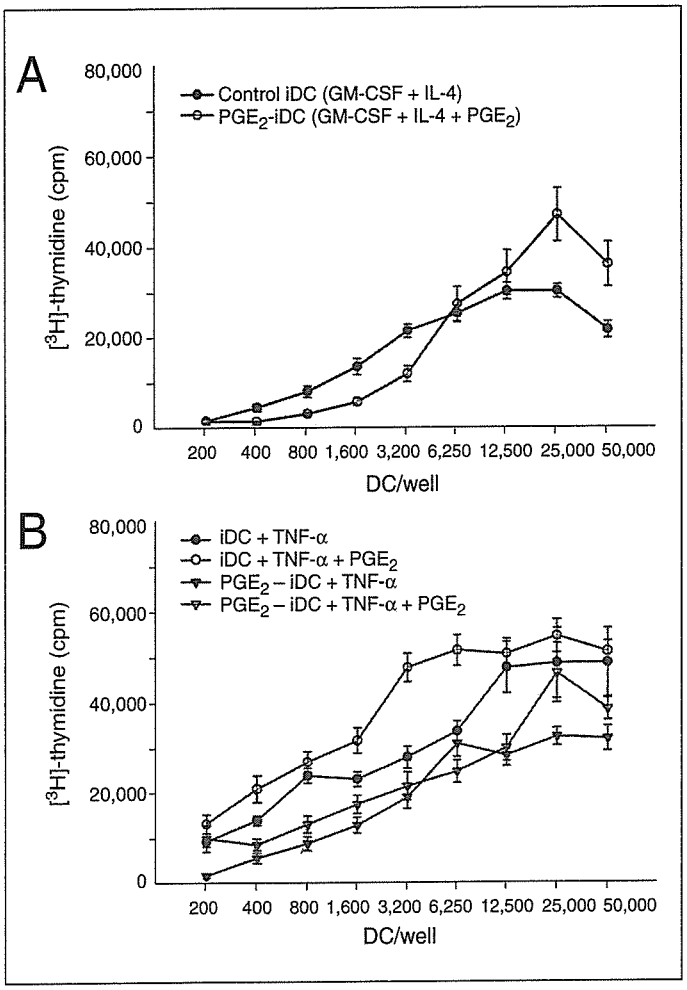
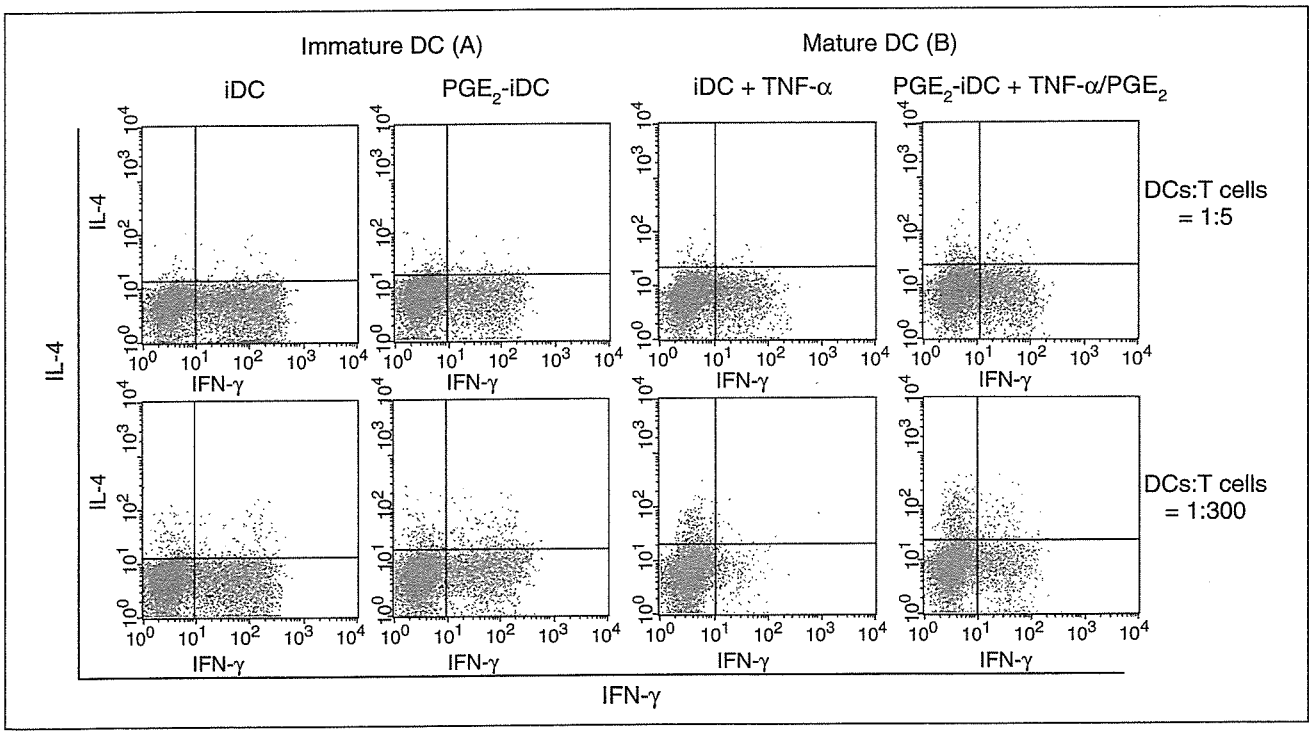


Figure 1. Allogeneic T-cell stimulatory capacity of immature DCs (A) and mature DCs (B) cultured with or without PGE_2. *$CD3^+$ cells (5×10^4 cells/well) isolated from healthy donors were cocultured with graded doses of irradiated DCs, and on day 5, [^{3}H]-methylthymidine was added 16 hours before measurement of the proliferative response. Data shown are the mean cpm (± SE) of triplicate cultures from four independent experiments.*

(Figs. 2 and 3). The addition of PGE_2 resulted in a lower IFN-γ production by naïve T cells, and removal of PGE_2 at maturation induced a complete recovery of IFN-γ secretion (Fig. 3). This suggests that, in the presence of PGE_2, immature or mature DCs induce naïve T cells to differentiate toward a Th1 response and that PGE_2 inhibits the Th1 polarization of naïve T cells. Additionally, T cells cocultured with immature or mature DCs at a low cell ratio (1:300) showed a greater production of IL-4 and a lower production of IFN-γ than those at a high cell ratio, regardless of the

Figure 2. Immature (A) and mature DCs (B) cultured in the presence of PGE_2 induced the differentiation of naïve $CD4^+CD45RA^+$ cells to a Th1 response at a high ratio of DCs/T cells (1:5) and to a Th1/Th2 response at a low ratio (1:300). *Naïve T cells were cocultured with DCs after being pulsed with staphylococcal exotoxins, expanded with the addition of 500 U/ml IL-2 from day 5, and harvested on day 14. Intracellular cytokine (IFN-γ and IL-4) concentrations were measured after restimulation with PMA and ionomycin for 4 hours on day 14. The data shown are from one representative experiment. Similar data were obtained in two other subgroups of mature DCs.*



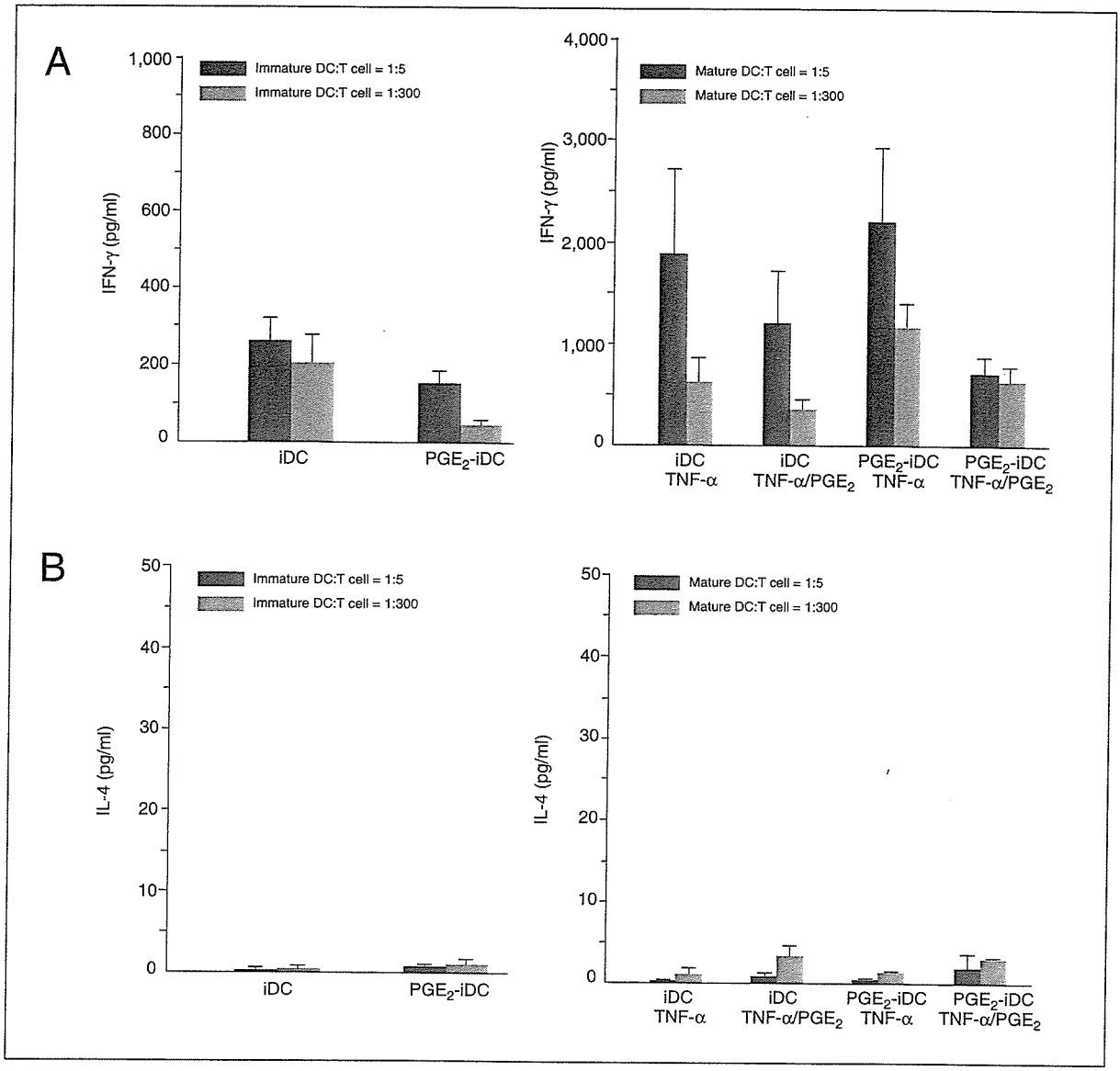


Figure 3. Measurement of IFN-γ (A) and IL-4 (B) by ELISA in supernatant (on day 14) of naïve CD4⁺CD45RA⁺ T cells stimulated by immature or mature DCs. *DCs under culture with PGE_2 induced naïve T cells to secrete high amounts of IFN-γ, although less than that secreted by control cells, but a low amount of IL-4 at a high ratio of DCs/T cells (1:5). However, naïve T cells at a low ratio (1:300) tended to have a lower IFN-γ level and a greater IL-4 level than those at a high ratio (1:5). Data shown are the mean ± SE from four independent experiments.*

presence of PGE_2. This finding suggests that naïve T cells are polarized toward both Th1 and Th2 responses at a low DC/T-cell ratio (Figs. 2 and 3).

Levels of IL-12 and IL-10 measured in the culture supernatant of immature or mature DCs were basically very low, with no difference between the presence or absence of PGE_2 (Fig. 4). In this study, IL-12 and IL-10 were measured in the basal state without any other stimulation. Therefore, this finding suggests that T-cell polarization by DCs in the basal state does not depend on IL-10 or IL-12.

Mature DCs Cultured with PGE_2 Did not Augment the Cytotoxicity of $CD8^+$ T Cells Against BEC-2 Target Cells

We compared the CTL responses of autologous $CD8^+$ T cells supported by mature DCs cultured with or without PGE_2, after the observation that DCs in the presence of PGE_2 induced naïve T-cell differentiation into a Th1 response. $CD8^+$ T cells that were stimulated with EBV-pulsed mature DCs showed stronger CTL responses against BEC-2 target cells, as measured by IFN-γ ELISA assay, than against Bamb-2 target cells. This stimulation depended on the effector-to-target ratio. However, the