Table 2 Patient-based results

	TP	FN	TN	FP	Sensitivity (%), 95% CI	Specificity (%), 95% CI	Accuracy (%), 95% CI
PET	12	3	12	3	80.0 59.8–100	80.0 59.8–100	80.0 67.2–92.8
PET/CT	14	1	14	1	93.3 80.7–100	93.3 80.7–100	93.3 84.4–100

TP true positive, *FN* false negative, *TN* true negative, *FP* false positive

Results

In 15 (50%) of the 30 patients, recurrence and distant metastasis were confirmed by pathological examinations ($n = 12$) and clinical follow-up study ($n = 3$). On patient-based analysis, PET/CT interpretation was true positive in 14 of the 15 patients with recurrence and true negative in 14 of the 15 patients without recurrence, whereas PET interpretation was true positive in 12 of the 15 patients with recurrence and true-negative in 12 of the 15 patients without recurrence. Thus, on patient-based analysis, the sensitivity, specificity, and accuracy of PET/CT were 93.3% (95% CI, 80.7%–100%), 93.3% (95% CI, 80.7%–100%), and 93.3% (95% CI, 84.4%–100%), respectively, whereas those of PET were 80.0% (95% CI, 59.8%–100%), 80.0% (95% CI, 59.8%–100%), and 80.0% (95% CI, 67.2%–92.8%), respectively (Table 2). Although PET/CT interpretation yielded higher diagnostic results than PET-alone interpretation, the difference was not statistically significant ($P = 0.479$ in sensitivity or specificity, $P = 0.134$ in accuracy; McNemar test) because of the small sample size.

On lesion analysis, PET/CT revealed only one false-negative case and one false-positive case, whereas PET revealed three false-negative cases and five false-positive cases (Table 3; Figs. 1, 2, 3). One false-negative PET/CT case was a missed para-aortic lymph node metastasis measuring 6 mm, which was subsequently confirmed by follow-up PET/CT and surgery. One false-positive PET/CT case was an over-diagnosed Th6 vertebra bone metastasis owing to focal moderate FDG accumulation at PET and osteolytic degenerative change at CT, which were confirmed not to be a malignancy by bone biopsy and follow-up MRI. Three false-negative PET cases comprised two cases of para-aortic lymph node metastases measuring 6 mm and 11 mm and one case of tiny lung metastases, smaller than 1 cm (Fig. 3). Five false-positive PET cases were as follows: one case of physiological FDG uptake in the intestine that was misinterpreted as peritoneal dissemination, one case of physiological FDG uptake in the intrapelvic vessels that was misinterpreted as pelvic lymph node metastasis, one case of physiological and reactive FDG uptake in the mediastinal and hilar lymph nodes that was misinterpreted as lymph node metastasis, one case of pulmonary pneumonia that was misinterpreted as lung metastasis, and one case of degenerative vertebra bone change that was misinterpreted as bone metastasis. In short, of the three false negatives with PET, PET/CT correctly identified two lesions as true positive, and of the five false positives with PET, PET/CT correctly identified four lesions as true negative.

Table 3 Lesion-based results

Site	TP	FN	TN	FP
Lung				
PET	3	1	25	1
PET/CT	4	0	26	0
Liver				
PET	2	0	28	0
PET/CT	2	0	28	0
Bone				
PET	1	0	28	1
PET/CT	1	0	28	1
Pleura and peritoneum				
PET	3	0	26	1
PET/CT	3	0	27	0
Supraclavicular LN				
PET	2	0	28	0
PET/CT	2	0	28	0
Mediastinal and hilar LN				
PET	2	0	27	1
PET/CT	2	0	28	0
Para-aortic LN				
PET	2	2	26	0
PET/CT	3	1	26	0
Pelvic LN				
PET	4	0	25	1
PET/CT	4	0	26	0
Inguinal LN				
PET	1	0	29	0
PET/CT	1	0	29	0

LN lymph node

Discussion

To our knowledge this is the first study to investigate the added diagnostic value of PET/CT over PET for diag-

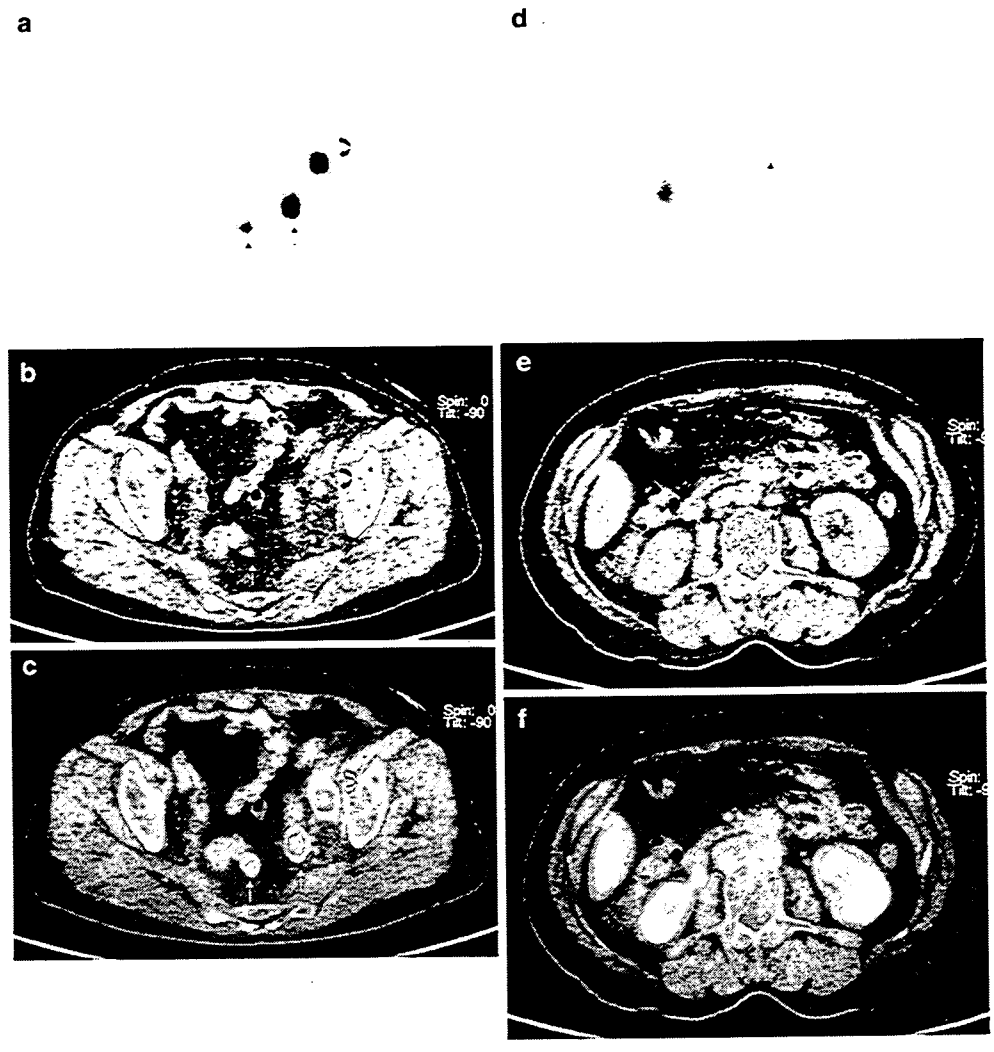

Fig. 1 A 73-year-old woman with initial International Federation of Gynecology and Obstetrics (FIGO) stage II b A with sigmoid colon mesenteric disseminations and para-aortic and pelvic lymph node metastases. Axial positron emission tomography (PET, **a**), computed tomography (CT, **b**), and PET/CT (**c**) showing two small sigmoid colon mesenteric disseminations (*arrows*) and a left internal iliac lymph node metastasis (*curved arrow*). Axial PET (**d**), CT (**e**), and PET/CT (**f**) showing para-aortic lymph node metastasis (*arrow*)

nosing recurrence of endometrial cancer. PET/CT tended to improve the restaging accuracy when compared with PET alone by slightly raising all of sensitivity, specificity, and accuracy. The difference, however, did not reach statistical significance, which might be attributed to the relatively small patient group.

Three groups have investigated the usefulness of FDG-PET for postoperative or post-therapy surveillance of patients with endometrial cancer. Belhocine et al. [16] performed 41 FDG-PET examinations in 34 women with previously treated endometrial cancer. They found the sensitivity, specificity, and accuracy to be 96%, 78%, and 90%, respectively. One false-negative result was microscopic lung metastases revealed by thoracic CT and three false-positive results were benign tumors, inflammatory post-therapy change, and physiological retention of the tracer in the urinary system or bowels. Saga et al. [17] performed 30 FDG-PET examinations in 21 postoperative patients with endometrial cancer and found that FDG-PET had a sensitivity of 100%, a specificity of 88%, and an accuracy of 93% with the help of anatomic information provided by CT and/or MRI. Four false-positive PET results comprised three cases of physiological uptake in the intestine that was misinterpreted as dissemination of lymph node metastases, and one case of heterogeneously increased uptake in the bone marrow following granulocyte-colony stimulating factor administration for chemotherapy-induced neutropenia that was misinterpreted as bone metastasis. Chao et al. [18] performed 60 FDG-PET examinations in 49 women with histologically confirmed endometrial cancer, among which 27 examinations were performed for primary staging and 33 for post-therapy surveillance or restaging on relapse. The sensitivity of FDG-PET alone or FDG-PET plus MRI/CT for detecting lesions overall was significantly higher than that of MRI/CT alone in the 60 scans. FDG-PET had a negative impact in three patients undergoing recurrence surveillance, or staging after

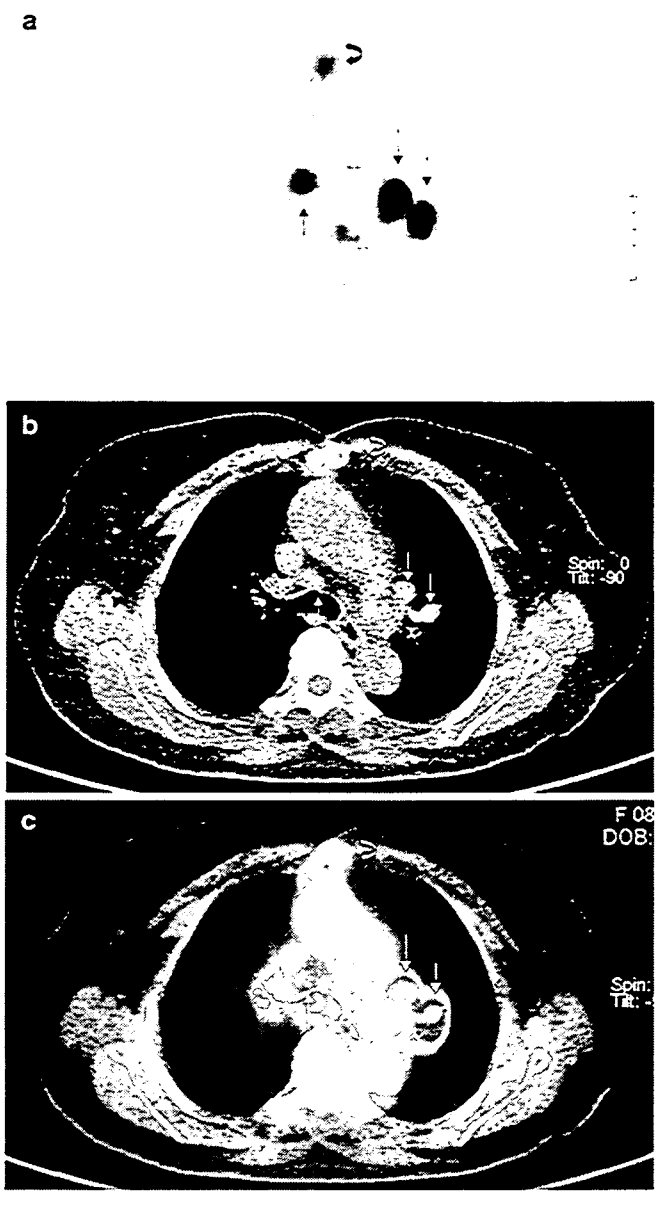

Fig. 2 An 82-year-old woman with initial FIGO stage III c with mediastinal and hilar lymph node metastases. Axial PET (**a**), CT (**b**), and PET/CT (**c**) showing mediastinal and hilar lymph node metastases (*arrows*)

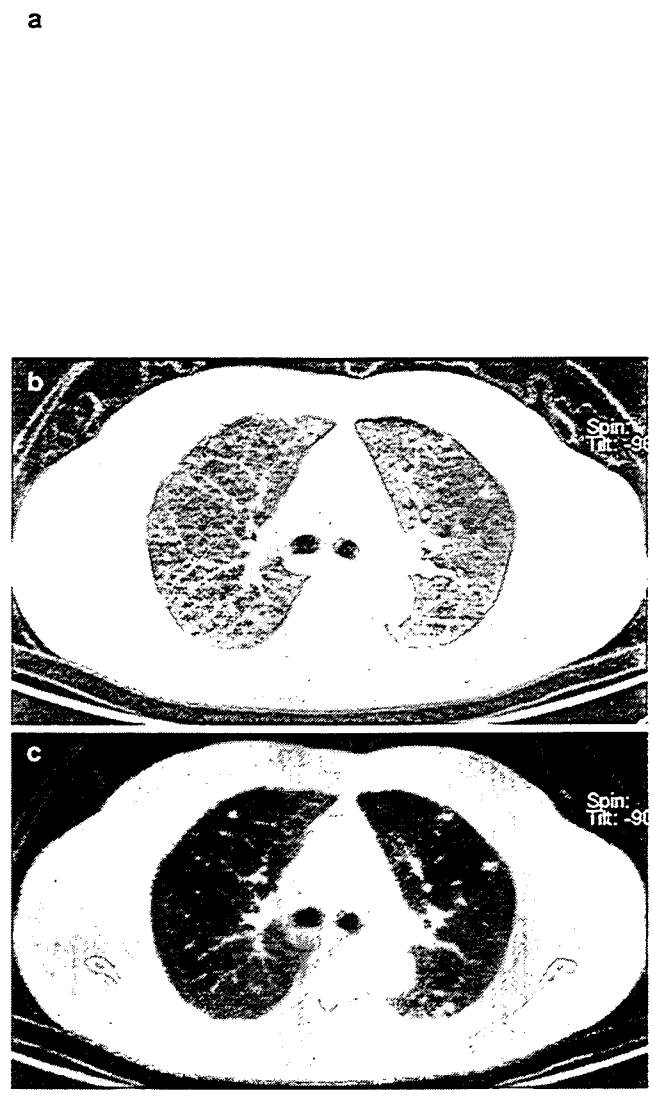

Fig. 3 A 67-year-old woman with initial FIGO stage III a with tiny lung metastases. Although axial PET (**a**) showing no abnormal FDG uptake in the lung, CT (**b**), and PET/CT (**c**) clearly showing many tiny lung metastases

recurrence: one false-positive result was a liver lesion and two false-negative results were peritoneal dissemination and liver metastasis.

Like these previous PET reports, PET interpretation in our series overdiagnosed benign inflammatory/infectious tissue and physiological uptake as recurrence and distant metastasis. This limited specificity of PET could partially be resolved by PET/CT acquiring both metabolic and anatomic imaging information in our series. Moreover, CT from PET/CT could detect tiny lung metastasis and para-aortic lymph node measuring 11 mm that PET missed. But a tiny para-aortic lymph node metastasis could not be detected not only by PET but also by PET/CT. PET or PET/CT can only detect the lymph nodes that have a certain volume of malignant cells sufficient to change the glucose metabolism, and neither of these modalities can detect micrometastasis. The spatial resolution of PET scans is insufficient for the detection of microscopic metastases to lymph nodes [19]. With a given spatial resolution of 4–6 mm with currently available PET and PET/CT systems, the detection of microscopic lesions remains challenging. Improving the spatial resolution and sensitivity of PET and PET/CT scanners and developing new, more specific radioactive tracers may help overcome this limitation in the future.

In this study, the CT component of PET/CT was low dose and did not use oral or intravenous contrast mate-

rial. Because the low-dose unenhanced CT from PET/CT is certainly not optimal for diagnostic interpretation and comparing PET/CT with the CT from PET/CT would have had little clinical relevance, CT alone from PET/CT was not interpreted in our study. Adding an oral contrast agent would possibly help to better delineate normal bowel activity and demonstrate pathological intra-abdominal activity (peritoneal implants). Use of an intravenous contrast agent can differentiate small lymph nodes from vessels, intestine, or the ureter, and correctly detect small liver metastasis, small peritoneal dissemination, and local recurrence at the vagina. A further PET/CT study with oral and intravenous contrast material is warranted to more precisely define its clinical role and accuracy for the detection of recurrent lesions.

This study had certain limitations. First, the number of patients with suspected endometrial cancer recurrence in our series was small. More studies are needed with a larger sample size to help verify the sensitivity and specificity of PET/CT. Second, the ideal gold standard for any analysis is the histological confirmation of the findings. However clinical follow-up is a valid way to evaluate diagnostic accuracy and response to therapy, and it would have been unethical to investigate all PET/CT-detected lesions by invasive procedures. Positive findings are easy to confirm, but negative findings only mean that we were unable to acquire positive findings during the follow-up period, making it uncertain as to whether the findings were truly negative. Third, conventional morphological imaging modalities including CT and MRI, which were used to detect recurrent lesions before PET/CT scan were not performed in all patients and we could not accurately compare the results of conventional imaging interpretation and PET/CT interpretation.

In conclusion, integrated FDG-PET/CT is a useful complementary modality for providing good anatomic and functional localization of sites of recurrence during follow-up of patients with endometrial cancer.

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Plasma levels of α-defensins 1–3 are an indicator of neutrophil activation in pregnant and post-partum women

Takayuki Okazaki[1], Yoriko Ota[1], Nobuhiro Yuki[2], Ayako Hayashida[1], Akiko Shoda[1], Masayoshi Nishikawa[1], Kyoko Oshima[1], Ichio Fukasawa[1], Hiroshi Watanabe[1] and Noriyuki Inaba[1]

[1]*Department of Obstetrics and Gynecology, and* [2]*Department of Neurology and Research Institute for Neuroimmunological Diseases, Dokkyo Medical University School of Medicine, Tochigi, Japan*

Abstract

Aim: In severe preeclampsia and septic shock, excessively activated neutrophils are thought to injure tissue irreversibly. On the other hand, mild neutrophil activation is known to occur during normal pregnancy. The objective of this study was to determine whether elevated plasma levels of α-defensins 1–3 could be used as an indicator of neutrophil activation in pregnant and post-partum women.

Methods: Defensin concentrations in 21 non-pregnant women and men, 184 normal pregnant women, and 55 post-partum women were quantified using an enzyme-linked immunosorbent assay (ELISA). The expression of the surface markers, CD11b and Toll-like receptor-4 (TLR-4), on the neutrophils were analyzed by flow cytometry in a cohort of subjects different from that used for the analysis of α-defensin levels.

Results: The concentrations of α-defensins were significantly higher in women that were in labor than in any of the other subjects. These levels diminished after delivery, but remained significantly elevated at one month post-partum. The expression of both CD11b and TLR-4 was significantly higher in women in labor compared to non-pregnant donors (controls). CD11b expression remained high on the third post-partum day, while TLR-4 expression fell to non-pregnant levels.

Conclusion: Our results suggest that there is a positive association between defensin levels and neutrophil activation in pregnant and post-partum women.

Key words: CD11b, defensin, labor, neutrophil, TLR-4.

Introduction

Neutrophils play an important role in the innate immune response to infection in mammals. These cells produce antimicrobial α-defensins 1–3 that are stored in specific, azurophilic granules and are released in response to infection.[1] Stimuli such as infection and injury induce the activation of neutrophils, which respond through phagocytosis and the release of antimicrobial peptides such as α-defensins 1–3. In addition to further activating the neutrophils themselves, these defensin molecules promote migration and phagocytosis by other phagocytic cells such as macrophages, and stimulate mast cells to release histamine, which increases vascular permeability and promotes the accumulation of more inflammatory cells. Finally, defensins, as well as other antimicrobial peptides increase the production of endothelial interleukin-8, which further promotes inflammation.[2]

Mild activation of neutrophils has also been noted during normal pregnancy in response to syncytiotrophoblastic, apoptotic debris that enters the maternal circulation.[3] Once labor begins, the amount of tissue debris that enters the maternal circulation increases

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Reprint request to: Dr Takayuki Okazaki, Department of Obstetrics and Gynecology, Dokkyo University School of Medicine, Kitakobayashi 880, Mibu, Shimotsuga, Tochigi 321-0293, Japan. Email: okazaki@dokkyomed.ac.jp

and induces a systemic inflammatory response.[4-8] The role played by neutrophils in this post-partum inflammatory process has not been investigated.

Preeclampsia, defined as high blood pressure and excessive protein levels in the urine after 20 weeks of pregnancy, is thought to be caused, in part, by the increased systemic activation of neutrophils,[9] poor placentation,[10] and, in more serious cases, maternal systemic inflammation.[11,12] In light of the reported association of activated neutrophils with this condition, an assessment of the degree of neutrophilic activation during pregnancy may be clinically useful in diagnosing preeclampsia in its early stages. In this study, we determined whether the measurement of plasma α-defensins 1–3 levels is useful in the assessment of the extent of neutrophil activation in normal pregnant and post-partum women.

Materials and Methods

The institutional ethical committee approved the entire protocol of this study.

Plasma concentration of α-defensins 1–3

Plasma samples were obtained from a total of 260 individuals after receiving their informed consent. The subjects included 21 healthy donors (the control group, 11 men and 10 non-pregnant women), 184 normal pregnant women ($n = 53$, 43, 52, 10 and 26 at 8–12 weeks gestation, 23–30 weeks gestation, 33–36 weeks gestation, 37–40 weeks gestation and not in labor, and 37–40 weeks gestation and in labor, respectively), and 55 women after term normal vaginal delivery ($n = 37$ and 18 within 1 week and at 1 month post delivery, respectively). Women 'in labor' were defined as women at the beginning of the active phase of first stage term labor. All of the pregnant women were outpatients at our clinic from June 2003 to January 2005. Exclusion criteria included a body temperature above 38°C, obvious symptoms of an infectious disease, and moderate to severe obstetric and systemic disorders, especially those accompanied by inflammation, such as diabetes, asthma, preeclampsia, or threatened premature delivery.

All venous blood samples were harvested under uniform conditions into heparinized tubes that were immediately centrifuged at $300 \times g$ for 30 min to separate out the plasma and to prevent the degranulation of neutrophils that would have occurred during blood clotting.[13] The concentration of defensins in these samples was assessed using an immunoassay as previously described.[14] Specifically, the plasma concentrations of α-defensins 1–3 were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Human HNP 1–3 ELISA Test Kit, HyCult Biotechnology b.v, Uden, the Netherlands) following the manufacturer's instructions. Each sample was diluted 1000-fold and each assay was run in duplicate in a microplate, which was read in a microplate reader (Biotrak II Visible Plate Reader, Amersham Biosciences Corp., Piscataway, NJ) at an optical density of 450 nm.

Neutrophil counts

Neutrophils were counted as part of routine check-ups that the 136 women underwent during and after their normal pregnancy. The blood samples used to obtain plasma were also used to quantify α-defensins 1–3 levels. Neutrophil counts were calculated by multiplying the total number of leukocytes by the percentage of neutrophils. The leukocyte counts and neutrophil fractions were obtained using an automated leukocyte differential system (CELL-DYN 4000, ABBOT JAPAN, Tokyo, Japan). The normal ranges for leukocyte counts and the neutrophilic fraction in our hospital have been set to $4.0–9.0 \times 10^9$/L and 37–73%, respectively.

Analysis of neutrophil surface markers

Venous blood was harvested from seven non-pregnant healthy women, seven healthy pregnant women in labor, and seven women three days after normal delivery. These subjects were different from those included in the analyses above. Neutrophils were isolated within 90 min of harvesting using a density gradient method with 2% dextran and Ficoll-Paque solution (Ficoll-Paque PLUS, density = 1.077, Amersham Biosciences Corp.). Expression of both CD11b and Toll-like receptor-4 (TLR-4) on the surface of neutrophils was determined using flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). A cell suspension of 5×10^5 cells in 500 μL of 0.1 M phosphate buffer solution (pH 7.2) was treated with 10 μL PE-antihuman monoclonal CD11b antibody (eBioscience Inc., San Diego, CA) at 4°C for 20 min or with 1 μL PE-antihuman monoclonal TLR-4 antibody (eBioscience Inc.) at room temperature for 15 min. PE-antimouse IgG2a monoclonal antibody (eBioscience Inc.) was used as the control antibody. The cells were washed and resuspended in 2 mL phosphate-buffered solution, then analyzed by flow cytometry. Neutrophil purity, assessed by size and granularity during flow cytometry, was greater

Table 1 Characteristics of this study and neutrophil counts

Groups	*n*	Age (y-o)	Neutrophil counts (10^9/L)
Healthy nonpregnant individuals		29.5 ± 3.9*	3.83 ± 1.30
Men	11		
Women	10		
Normal pregnancy			
8–12 weeks (1st trimester)	53	29.6 ± 5.2*	5.62 ± 1.56
23–30 weeks (2nd trimester)	43	29.7 ± 4.6*	6.43 ± 1.39
33–40 weeks (3rd trimester)	62	30.6 ± 5.1*	5.84 ± 1.93
37–40 weeks (in trimester)	26	32.1 ± 5.3*	8.71 ± 1.35**
Normal puerperium			
<1 week	37	30.8 ± 6.2*	6.98 ± 2.17
1 month	18	29.0 ± 5.9*	4.21 ± 0.76

*$P = 0.57$ (one-way ANOVA); **$P < 0.05$ versus all other groups (Scheffe's F test); Data is presented as the mean ± standard deviation.

than 91%. Twenty thousand cells were analyzed in each assay using CellQuest software (Becton Dickinson).

Statistical analyses

After performing a Bartlett test for homogeneity of variance, one-way ANOVAS were used to evaluate whether there were any age differences among the groups. The significance of differences in the concentrations of α-defensins 1–3, neutrophil counts and the expression of neutrophil surface markers among groups was determined using a Kruskal–Wallis test for non-parametric analysis of variance followed by Scheffe's *F*-test. $P < 0.05$ was considered statistically significant.

Results

Plasma concentrations of α-defensins 1–3

There were no significant differences in the mean age of our 260 subjects among the groups (Table 1). The plasma α-defensin 1–3 concentrations for each of our experimental groups are illustrated graphically in Figure 1. There was no significant difference in the median defensin concentrations between non-pregnant healthy women and healthy men (median ± SEM = 45.0 ± 39.0 vs 35.8 ± 26.3 ng/mL, respectively). The median value for all non-pregnant individuals was 39.0 ± 8.1 ng/mL, while the median concentrations for pregnant women at 8–12 weeks gestation, 23–30 weeks gestation, 33–36 weeks gestation, 37–40 weeks gestation and not in labor, and 37–40 weeks gestation and in labor were 38.9 ± 14.3 ng/mL, 71.8 ± 21.0 ng/mL, 80.0 ± 17.5 ng/mL, 96.8 ± 21.1 ng/mL, and 275 ± 85.6 ng/mL, respectively. The concentrations of α-defensins 1–3 in the plasma of women who had delivered their baby

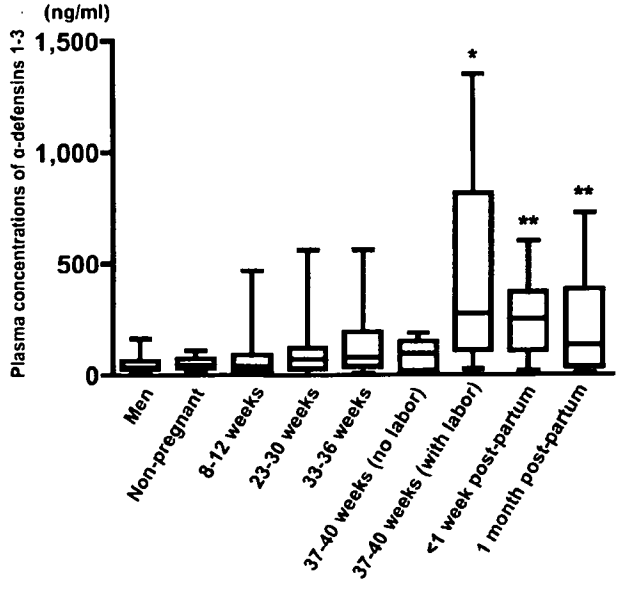

Figure 1 Median concentration and dispersion of plasma concentrations of α-defensins 1–3 during normal pregnancy and post-partum. Blood samples were obtained from non-pregnant healthy donors, normal pregnant women (8–12 weeks gestation, 23–30 weeks gestation, 33–36 weeks gestation, 37–40 weeks gestation and not in labor, and 37–40 weeks gestation and in labor), and women after normal term vaginal delivery (within 1 week and at 1 month post delivery). The error bars indicate the standard error of mean. *$P < 0.001$ versus all other groups, **$P < 0.05$ versus control (Scheffe's *F*-test).

within 1 week and 1 month earlier were 251 ± 34.2 ng/mL and 134 ± 60.8 ng/mL, respectively. The median plasma defensin concentration was significantly higher for women in labor than for any other group, and levels remained significantly higher than in the control group at 1 month post-partum.

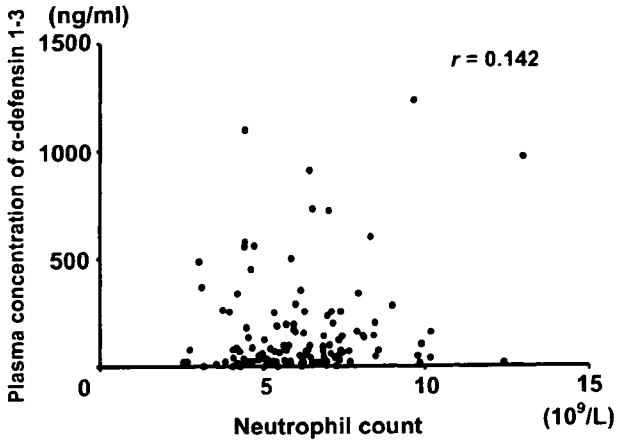

Figure 2 Scatter plot of the concentration of plasma α-defensins 1–3 and neutrophil counts during pregnancy and post-partum. Neutrophils were counted as part of routine check-ups that the 136 women underwent during and after their normal pregnancy using the same blood samples from which plasma was obtained to quantify their α-defensins 1–3 levels. $p = 0.105$ (Spearman's correlation coefficient by rank test).

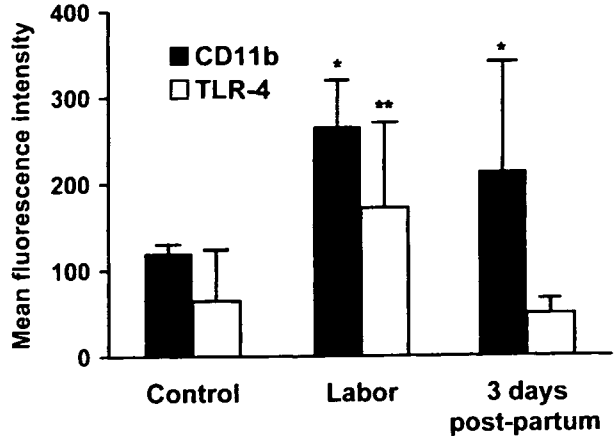

Figure 3 Quantification of surface marker expression on neutrophils. The solid bar indicates CD11b and the open bar indicates Toll-like receptor-4 (TLR-4). Blood samples were obtained from non-pregnant healthy women (controls), normal pregnant women in labor, and women at 3 days post-partum. The error bars indicate standard deviation. $^{*}P < 0.05$ versus control, $^{**}P < 0.05$ versus all of the other groups (Scheffe's *F*-test).

Correlation between α-defensins 1–3 plasma concentration and neutrophil counts

The same samples that were used to quantify defensin levels were used to count the number of venous blood neutrophils. Our results showed that women in labor had significantly higher neutrophil counts than subjects in any of the other groups (Table 1). After delivery, neutrophil counts fell quickly, reaching non-pregnant values by one month post-partum. There was no correlation between defensin concentrations and neutrophil counts (Fig. 2).

Expression of TLR-4 and CD11b on neutrophils

The mean fluorescence intensity of TLR-4 expression was 64 in the control group, but 172 in women in labor ($P < 0.05$). On the third post-partum day, TLR-4 expression fell to 49, which was not significantly different from controls. The mean fluorescence intensity of CD11b expression was significantly elevated in women in labor (266) compared to that in controls (120; $P < 0.05$). On the third day post-partum, CD11b expression remained significantly elevated (214) (Fig. 3).

Discussion

We have found no published reports detailing the levels of α-defensin during and after pregnancy. Evidence suggests that the number of neutrophils increases steadily during pregnancy, decreases after delivery, and reaches non-pregnant levels by about 1 month post-partum.[9,14] Since α-defensins 1–3 are stored in neutrophilic granules and are only released by activated neutrophils, we hypothesized that the levels of these compounds in plasma may correlate with neutrophil activation rather than the number of neutrophils in peripheral blood. Elevations in the numbers of neutrophils during normal pregnancy are thought to be due to delayed apoptosis.[15] TLR-4 was reported to be the principal regulator of neutrophil survival, with TLR-4-mediated neutrophil survival dependent upon signaling via the NFκB and mitogen-activated protein kinase cascades. TLR-4-activated monocytes were also found to play an essential role in delaying neutrophil apoptosis by releasing neutrophil survival factors.[16]

Although neutrophil CD11b expression has been reported to be increased in normal pregnant women[17] and the plasma level of elastase, granular components derived from azurophilic granules of neutrophils, is elevated in normal pregnancy,[18] there were no significant differences observed in the plasma α-defensin 1–3 concentrations between normal pregnant groups without labor and the non-pregnant group. The reasons why the plasma α-defensin 1–3 concentrations

were different from previous reports of CD11b expression and elastase in non-pregnant and normal pregnancy without labor could not be determined because the correlations between defensins and CD11b expression or elastase concentration were not analyzed in this study. The large dispersion of the plasma α-defensin 1–3 concentrations in normal pregnant groups may explain the inconsistency with previous reports.

Our data failed to reveal a correlation between plasma α-defensin levels and neutrophil counts during pregnancy and labor. We hypothesized that plasma α-defensin levels may reflect neutrophil activation, but not overall numbers. We evaluated the intensity of expression of CD11b, an established marker of neutrophil activation,[19–21] and TLR-4, a marker of delayed apoptosis in neutrophils, at the onset of labor and on the third post-partum day. CD11b expression and plasma α-defensin levels were significantly elevated in pregnant women in labor and in women in the early post-partum days compared to controls. TLR-4 expression was also significantly elevated during labor, but fell to non-pregnant levels by the third post-partum day. These results suggest that plasma α-defensin levels correlate with neutrophil activation rather than delayed apoptosis, i.e. the number of neutrophils.

Systemic inflammatory response syndrome (SIRS) is a critical condition that is often observed in patients in the intensive care unit who are suffering from septic shock, serious burn or other types of injury, or acute pancreatitis.[22] Both SIRS and severe preeclampsia are characterized by several clinical signs that include acute renal failure, intravascular disseminated coagulation (DIC), acute respiratory distress, and over-activation of neutrophils.[15] Neutrophils are also thought to help trigger labor, which in this context can be thought of as resulting from an inflammatory process, especially since they seem to accumulate in the lower rather than upper segment of the myometrium.[4] Thus, a large amount of α-defensin 1–3 would probably be released in severe preeclampsia and contribute to the cervical ripening that occurs during spontaneous labor and in cases of threatened premature delivery.

In conclusion, our results suggest that there is a positive association between defensin levels and neutrophil activation in pregnant and post-partum women. Further work is required to determine the significance of this data in predicting clinical outcomes in patients with preeclampsia or threatened premature delivery. It will contribute to resolve these complications during pregnancy.

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Prediction of the Efficacy of Antiviral Therapy for Hepatitis C Virus Infection by an Ultrasensitive RT-PCR Assay

Ei Kinai,[1] Hideji Hanabusa,[2] and Shingo Kato[3]*

[1]*Department of Pediatrics, Keio University Hospital,, Shinjuku-ku, Tokyo, Japan*
[2]*Department of Haematology, Ogikubo Hospital, Suginami-ku, Tokyo, Japan*
[3]*Department of Microbiology and Immunology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan*

The efficacy of interferon therapy for hepatitis C virus (HCV) infection improved remarkably. However, virologic relapse occurs in a substantial proportion of patients with virologic response (defined as an HCV RNA level below 50 IU/ml at the end-of-treatment). A highly sensitive RT-nested PCR assay capable of detecting almost a single copy of HCV RNA and a real-time RT-PCR assay to quantify HCV RNA down to 120 copies per ml were developed. The RT-nested PCR assay showed that 1 IU of HCV RNA is equivalent to 12.2 copies. For 28 patients with virologic response (12 relapsers and 16 sustained virologic responders), week-4 and end-of-treatment plasma samples were retested. At week 4, HCV RNA was detected by the RT-nested PCR and qualitative COBAS Amplicor HCV version 2.0 in 8/9 (89%) and 6/9 (67%) samples from relapsers, and in 4/16 (25%) and 2/16 (13%) samples from sustained virologic responders, respectively. End-of-treatment samples with HCV-negative by the qualitative COBAS Amplicor were positive by the present assay in 4/12 (25%) of relapsing patients and 0/16 (0%) of sustained virologic responders. The viral levels detected by the present assay in the Amplicor-negative samples were 3.5–17.3 copies/ml, which is below the detection limit of COBAS Amplicor. In conclusion, the highly sensitive RT-nested PCR assay can predict sustained virologic response at week 4 and virologic relapse at the end-of-treatment more accurately than COBAS Amplicor, suggesting its usefulness in monitoring antiviral therapy for HCV infection. ***J. Med. Virol. 79:1113–1119, 2007.*** © 2007 Wiley-Liss, Inc.

KEY WORDS: hepatitis C virus; ultrasensitive RT-PCR; accurate prediction; residual HCV RNA

INTRODUCTION

Hepatitis C Virus (HCV) chronically infects over 170 million people worldwide. After acute infection, approximately 15% to 25% of infected individuals overcome the virus with resolution of the infection, while the remainders become chronically infected. Of individuals with chronic infection, 10% to 20% progress and thereafter 1% to 5% develop hepatocellular carcinoma [Cohen, 1999]. In Japan, the number of HCV-infected patients is estimated to be 3 million in a population of 120 million. It should be noted that over 90% of hemophilia patients had been infected with HCV and about 40% had been co-infected with human immunodeficiency virus from contaminated blood products in the early 1980s. They are now facing an increasing risk of developing end-stage liver disease.

Recently, treatment for chronic hepatitis C has made remarkable progress. Current protocols of combination therapy of peginterferon plus ribavirin have achieved high rates (40–63%) of sustained virologic response [Manns et al., 2001; Fried et al., 2002; Hadziyannis et al., 2004; Torriani et al., 2004], defined as a serum HCV RNA < 50 IU/ml at week 24 after the end-of-treatment. Since it was reported that patients who did not achieve an early virologic response (EVR), defined as a serum HCV RNA < 50 IU/ml at week 12, did not respond to further therapy [Davis et al., 2003], the consensus guidelines have recommended that therapy be discontinued for patients who did not achieve EVR [European Association for the Study of the Liver, 1999; National Institutes of Health Consensus Development Conference, 2002; Strader et al., 2004]. Moreover, to avoid side effects and save treatment costs, early identification of nonresponse or relapse is increasingly required. Although rapid virologic response, defined as a serum HCV RNA < 50 IU/ml at week 4, was reported to be highly associated with sustained virologic response, virologic relapse occurred at various rates (9–27%)

*Correspondence to: Shingo Kato, Department of Microbiology and Immunology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.
E-mail: skato@sc.itc.keio.ac.jp

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among patients who achieved rapid virologic response [Jensen et al., 2006]. This may be explained by the fact that the detection limit of 50 IU/ml is not low enough to identify the patients with strong early virologic suppression that leads to sustained virologic response.

Several studies using highly sensitive HCV assays have shown that virologic relapse can occur in patients with a low level of residual HCV RNA at the end-of-treatment, which cannot be detected by COBAS Amplicor HCV version 2.0 (Roche Molecular diagnostics, Pleasanton, CA) [Sarrazin et al., 2001; Watkins-Riedel et al., 2004; Desombere et al., 2005; Gerotto et al., 2006]. However, early virologic assessment has not been fully evaluated with such highly sensitive assays. To evaluate accurately the relationships between EVR and sustained virologic response, and between the residual HCV RNA at the end-of-treatment and virologic relapse, a highly sensitive reverse transcription (RT)-nested PCR assay that detect almost a single copy of HCV RNA and a real-time RT-PCR assay with a wide quantitation range were developed. These techniques were used to retest plasma samples from patients with relapse or sustained virologic response who had virologic responses at the end-of-treatment.

MATERIALS AND METHODS

Patients and Samples

Plasma samples were collected from 28 patients who had been treated with interferon therapy at Ogikubo Hospital (Tokyo, Japan) and achieved virologic responses at the end-of-treatment; 12 relapsers and 16 sustained virologic responders. All patients were male hemophiliac patients and had been infected with HCV through contaminated blood products. The characteristics of patients are shown in Table I. There were no significant differences in age, sex, HIV-1 co-infection or alanine aminotransferase levels between relapser and sustained virologic responders groups. Although the patients with genotype 1 infection appeared to have a higher rate of relapse, a statistical difference was not obtained ($P = 0.13$). All plasma samples were stored at −60°C until analysis.

RNA Extraction

Total RNA was extracted from 500 µl of plasma using a QIAamp UltraSens Virus Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations with some modifications. Briefly, plasma was diluted with 500 µl of phosphate buffered saline and centrifuged at 18,000 rpm (35,000*g*) for 60 min. Most of the supernatant was discarded and the residual 30 µl and precipitate were resuspended with 300 µl of Buffer AR containing 5.6 µl of carrier RNA preheated at 60°C. The mixture was incubated for 10 min at 60°C using a mixing incubator. Thereafter, RNA was purified as recommended.

Reverese Transcription-Nested PCR

Primers. Reverse transcription (RT)-nested PCR was performed using two sets of primers targeting a region of the 5′ untranslated region of HCV. Reverse transcription (RT) was carried out with primer KC12, 5′-CTCGCAAGCACCCTATCAGGCAG-3′ (nucleotides (nt) 276–299, identical to primer KY78 designed by Roche Molecular Systems) [Young et al., 1993]. The primer pair for the first-round PCR consisted of forward primer KC11, 5′-CTCCCCTGTGAGGAACTACTGTCT-3′ (nt 24–51), and reverse primer KC12. The primer pair for the second-round PCR consisted of forward primer KC13, 5′-TCCCGGGAGAGCCATAGTG-3′ (nt 115–133) and reverse primer KC14, 5′-TCCAAGAAAGGACCC-3′ (nt 176–196).

RT-nested PCR. RT was carried out in a 20-µl solution of PCR Buffer II (10 mM Tris–HCl, 50 mM KCl), 3 mM $MgCl_2$, 10 mM of DTT, 0.5 mM of each dNTP, 0.5 µM of RT primer (KC12), 0.5 U of RNase inhibitor (Promega, Madison, WI), and 2.5 U of SuperScript III (Invitrogen, Carlsbad, CA). The mixture was incubated for 5 min at 60°C, 5 min at 55°C, 5 min at 50°C, and then 5 min at 45°C. A GeneAmp PCR system 9700 thermocycler and thin-walled MicroAmp reaction tubes (Applied Biosystems, Foster City, CA) were employed for RT and PCR. The first-round PCR was carried out using the whole RT product in a final volume of 50 µl containing 1× PCR Buffer II, 2.5 mM $MgCl_2$, 200 µM

TABLE I. Patient Characteristics

Category	Virologic relapsers	Sustained virologic responders
Number of cases	12	16
Age, median (in years) (range)	35 (24–69)	32 (20–66)
Sex, male/female	12/0	16/0
Genotype[a]		
1	5 (42%)	0 (0%)
2	2 (17%)	2 (40%)
3	5 (42%)	2 (40%)
4	0 (0%)	1 (20%)
HIV co-infection		
HIV positive	6 (50%)	6 (38%)
HIV negative	6 (50%)	10 (62%)
ALT level, median (in U/L) (range)[b]	115 (29–264)	120 (59–708)

[a]Of 16 cases with sustained virologic response, genotype was determined in five cases.
[b]ALT, Alanine aminotransferase.

each dNTP, 1 μM KC11 and KC12 primers, and 1.25 U of AmpliTaq DNA polymerase. The mixture was incubated for 2 min at 97°C to facilitate denaturation, then PCR amplification was performed with 5 cycles of 5 sec at 97°C (denaturation), 15 sec at 48°C (annealing), and 15 sec at 60°C (extension), 25 cycles of 5 sec at 94°C and 15 sec at 60°C, and a final extension step of 5 min at 72°C. One microliter of the first-round PCR product was applied to the second-round PCR in a 50-μl solution containing the same components as the first-round PCR except for primers KC13 and KC14 instead of KC11 and KC12. PCR amplification was performed with preincubation of 2 min at 94°C, 5 cycles of 5 sec at 94°C, 15 sec at 48°C, and 15 sec at 60°C, 25 cycles of 5 sec at 94°C and 15 sec at 60°C, and a final extension step of 5 min at 72°C. The final PCR product was analyzed by electrophoresis in 2% agarose gel containing 0.5 μg/ml ethidium bromide.

Evaluation of Extraction Efficiency

Extraction efficiency was evaluated by comparing the HCV RNA copy counts of seven samples (one sample of genotype 1a, two genotype 1b, one genotype 2a, one genotype 2b, one genotype 3a, and one genotype 4) determined by Poisson quantitation (described below) of HCV RNA in plasma and purified RNA obtained from plasma using the modified RNA extraction protocol.

Poisson quantitation of purified RNA. The HCV RNA concentration was determined by statistical analysis of the results of RT-nested PCR of the sample diluted to near endpoint. First, serial 10-fold dilutions of the extracted RNA solution were assayed with RT-nested PCR as described above. Then, the diluted solution that had conferred the second to last positive band was serially diluted twice and tested with RT-nested PCR. Finally, the RNA solution diluted at the ratio by which the last positive signal was given in a series of twofold dilutions was assayed in 20 replicates. Assuming that a single copy of HCV RNA is detectable, the HCV concentration can be calculated from the proportion of negative reactions (P_0) using the null class equation of the Poisson distribution [Simmonds et al., 1990]. The formula is HCV RNA concentration = $-\ln P_0/V \times d$ (copies/μl), where V and d are the sample volume (μl) assayed and the dilution ratio, respectively. In these experiments, RNA Diluting Buffer (50% glycerol, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.05% Sarkosyl, 10 μg/ml poly A) was used to ensure no loss of RNA during the dilution procedures. We call the above procedure "Poisson quantitation."

Direct poisson quantitation of plasma RNA. Sample plasma was diluted with Virus Lysis Buffer (50% glycerol, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.05% Sarkosyl, 10 μg/ml poly A, 2 mM DTT, and 0.4 U/μl RNase inhibitor). Diluted samples were quantified by Poisson quantitation without RNA extraction. This method can quantify the absolute copy number of HCV RNA in plasma, since it is not affected by loss of RNA during the purification procedure. It was confirmed that contamination of less than 0.1 μl of plasma in one reaction did not affect quantitation of HCV RNA in this RT-PCR condition.

HCV RNA Real-Time RT-PCR

RT was carried out using a high-capacity cDNA Archive kit (Applied Biosystems) in a final volume of 10 μl containing 5 μl of RNA sample solution and 0.3 μM of KC12 primer. The RT reaction was carried out with a GeneAmp PCR system 9700 for 30 min at 48°C, followed by reverse transcriptase inactivation for 5 min at 95°C. The PCR reaction was carried out in a final volume of 50 μl containing 10 μl of the RT product, TaqMan buffer (Applied Biosystems), 5 mM $MgCl_2$, 400 μM dUTP, 200 μM dATP, dCTP, dGTP (each), 0.025 U/μl AmpliTaq Gold, 0.01 U/μl AmpErase UNG, 0.3 μM forward primer (KC13), and reverse primer (KC14). For signal detection, 0.1 μM MGB probe 5′-CTGCGGAACCGGTG-3′ (KCP) was used. The PCR amplification was performed using Sequence Detection Systems 7700 (Applied Biosystems). The temperature settings were: uracil-*N*-glycosylase incubation for 2 min at 50°C; AmpliTaq Gold activation for 10 min at 95°C; 40 cycles of 15 sec at 95°C; and 1 min at 60°C. Measurements of standard and unknown samples were done in triplicate. After amplification, real-time data acquisition and analysis were performed with the accompanying software. For the standard HCV RNA, we used an RNA solution purified from the plasma of one patient with genotype 1b, which is the most common genotype among Japanese patients and quantified by Poisson quantitation.

Detection and Quantitation of HCV RNA in Clinical Samples

From 28 HCV-infected patients who achieved a viral response with antiviral treatment (12 relapsers and 16 sustained virologic responders), 53 stored plasma samples at week 4 and at the end-of-treatment (9 week-4 samples and 12 end-of-treatment samples from relapsers, and 16 week-4 samples and 16 end-of-treatment samples from sustained virologic responders) were retested by the RT-nested PCR and real-time RT-PCR assays developed in this study. One tenth of the RNA purified from plasma samples as described above was subjected to in-house RT-nested PCR in 10 replicates. Plasma in which HCV RNA was detected in nine or more reactions was quantified by real-time RT-PCR, because a reliable HCV RNA value cannot be obtained for these samples by Poisson quantitation. The results of the RT-nested PCR and COBAS Amplicor HCV v2.0 assays were tested by Fisher's exact probability test with respect of the outcome.

RESULTS

Extraction Efficiency

The efficiency of HCV RNA extraction in the modified protocol of QIAamp UltraSens Virus kit was estimated by comparing the quantities of HCV RNA in seven plasma samples and in their extracted RNA, involving

six different HCV genotypes. The ratios ranged from 50% to 107% with a median of 89%.

Sensitivity of RT-Nested PCR

To evaluate the sensitivity of the RT-nested PCR, the World Health Organization international standard for HCV RNA (NIBSC code 96/798) was quantified by Poisson quantitation. In a total of 4 runs, HCV RNA was detected in 24 reactions of 34 replicates. This result showed that 1 IU was equivalent to 12.2 amplifiable copies (range based on SD, 9.9–15.3 copies).

Linearity, Reproducibility, and Genotype Reactivity of Real-Time RT-PCR

The data obtained for drawing the standard curves in the real-time RT-PCR assay were statistically analyzed to assess the precision within each run (intra-assay) and between runs (inter-assay). The standard curves were obtained with 10-fold dilutions of the RNA standard, ranging from 5 to 5,000 copies per reaction (equivalent to 120–120,000 copies/ml), and analyzed in triplicate in a total of four independent experiments. The results of the analysis are summarized in Table II. The standard deviation of the intra-assay on the basis of C_T values ranged from 0.05 to 0.74 cycles, even including samples with only 5 copies per reaction, and that of the inter-assay ranged from 0.70 to 1.42 cycles.

To overcome the variation of nucleotide sequences of 5′ untranslated region among HCV genotypes, we used the most conserved region for designing PCR primers. To evaluate the genotype reactivity of the real-time RT-PCR, obtained values of HCV RNA were compared with those determined by Poisson quantitation for HCV strains of various genotypes. There was very good linearity between the real-time RT-PCR and Poisson quantitation regardless of genotype (Fig. 1). The coefficient of variation ranged from 7.2% to 30.8% for all quantified points and all genotypes.

Detection and Quantitation of HCV RNA in Stored Plasma

From 28 patients who achieved virologic responses at the end-of-treatment, 53 plasma samples at week 4 or at the end-of-treatment were retested by the RT-nested PCR. The detection rates, statistical tests of week-4 and end-of-treatment samples, and the predictability of sustained virologic response by the undetectability of HCV RNA and relapse by the detection of HCV RNA are shown in Table III. In testing week-4 samples, the RT-nested PCR assay detected HCV RNA in 8 of 9 (89%) available samples from relapsers, and in 4 of 16 (25%) from sustained virologic responders ($P = 0.003$), whereas COBAS Amplicor detected 6/9 (67%) from relapsers and 2/16 (13%) from sustained virologic responders ($P = 0.01$). Among the 13 patients who were HCV RNA-negative at week 4 by the RT-nested PCR assay, 12 (92%) achieved sustained virologic response, compared to 14 of the 17 (82%) who were negative by COBAS Amplicor. In testing end-of-treatment samples, 4 of 12 (33%) Amplicor-negative samples from relapsers were HCV-positive by the RT-nested PCR assay, whereas 0 of 16 end-of-treatment samples from sustained virologic responders were HCV-positive ($P = 0.02$). Among the four patients who were HCV RNA-positive at the end-of-treatment by the RT-nested PCR assay, four (100%) had virologic relapse.

The viral load determined by Poisson quantitation in eight Amplicor-negative samples (six samples from relapsers and two samples from sustained virologic responders) were within 3.5–17.3 copies/ml (Table IV), which was below the detection limit of current highly sensitive assays such as transcription-mediated amplification.

DISCUSSION

This study demonstrates that an ultrasensitive HCV assay can identify both sustained virologic response and relapse in patients with high probabilities. As previously shown, the more sensitive the assay used for the quantitation of HCV RNA, the more predictive the measurements are of virologic relapse after discontinuation of therapy [Sarrazin et al., 2001; Desombere et al., 2005]. The currently developed highly sensitive HCV assay based on RT-nested PCR has an ability to detect almost a single copy of HCV RNA, which was achieved by a stepwise declining temperature in reverse transcription and a lower annealing temperature of 48°C for the first five PCR cycles. To assess the sensitivity of this assay, equivalency between the international unit and the amplifiable copy number of HCV RNA was determined using the WHO international standard. The international unit was first determined in 1998 based on a mean of measurements for the WHO international standard that were quantified by 22 multinational laboratories using various quantitation methods in 1998 [Saldanha et al., 1999], therefore, it is not yet clear how many HCV virions are equivalent to 1 IU.

TABLE II. C_T Values With RNA Dilutions for Standard Curve (Genotype 1b)

Copies/reaction	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Inter-assay (std dev)
5,000	22.20 (0.05)	23.60 (0.23)	22.30 (0.12)	21.98 (0.15)	0.70
500	26.54 (0.28)	27.59 (0.35)	25.90 (0.21)	25.39 (0.12)	0.88
50	30.99 (0.21)	31.35 (0.48)	29.14 (0.16)	28.55 (0.11)	1.26
5	35.46 (0.74)	35.40 (0.64)	33.27 (0.60)	32.60 (0.28)	1.42

Serially diluted samples were analyzed in triplicate in each experiment. Good consistency (intra-assay) and reproducibility (inter-assay) of the real-time PCR are shown in this table.

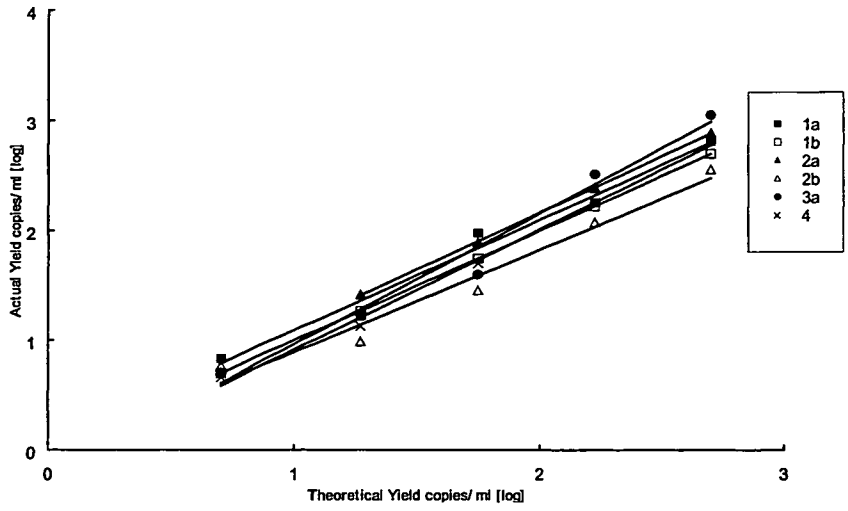

Fig. 1. Linearity of yield with RNA dilutions isolated from genotype 1a, 1b, 2a, 2b, 3a, and 4 samples. A sample from each genotype was serially diluted, and quantified by real-time RT-PCR. Actual yield copy counts were obtained from 10-fold diluted RNA samples by real-time RT-PCR, and theoretical yield copy counts were obtained from the dilution based on Poisson quantification using our RT-nested PCR.

Previously, conversion factors of international unit and amplifiable copy were determined at 2.75 and 5.2 copies/IU by Puig et al. [2002] and Gorrin et al. [2003], respectively. The result of 12.2 copies/IU in this study, which was obtained by analyzing the Poisson distribution of clear positive reactions at endpoint dilution of the WHO international standard, was considerably higher than the previous values. Taken together with a high efficiency (89%) of extraction of HCV RNA from plasma, the present assay is considered to have the ability to detect almost a single copy of HCV RNA in plasma samples.

The present qualitative RT-nested PCR assay was compared with the COBAS Amplicor HCV version 2.0 with a lower detection limit of 50 IU/ml for 53 plasma samples from 28 patients receiving antiviral therapy that were collected at week 4 or the end-of-treatment. While COBAS Amplicor detected HCV RNA in 8 samples, the present assay detected HCV RNA in 16 samples (all of 8 Amplicor-positive samples and 8 of 45 Amplicor-negative samples), providing evidence that the assay is more sensitive for the detection of HCV RNA in clinical samples than COBAS Amplicor version 2.0. Furthermore, regarding prediction of relapse or sustained virologic response after treatment, the present assay is more predictive than COBAS Amplicor at week 4 ($P = 0.003$ vs. $P = 0.02$) as well as at the end-of-treatment ($P = 0.02$ vs. $P = 1$). It is most likely that this difference is explained by a lower detection limit of the present assay. As mentioned above, the RT-nested PCR assay is approaching the ability to detect a single copy of HCV RNA. Therefore, when a 0.5 ml plasma sample is used, the sensitivity of the assay can be calculated from the equation for the Poisson distribution as 5 copies/ml at 95% detection, which is lower than 50 IU/ml for COBAS Amplicor and 25–50 copies/ml for transcription-mediated amplification assay (VERSANT HCV RNA Qualitative Assay) [Sarrazin et al., 2001].

TABLE III. Comparison of the Detection Rates of HCV RNA by Our in-House RT-Nested PCR at Week 4 and the End-of-Treatment, and COBAS Amplicor HCV Version 2.0 at Week 4

	In-house RT-nested PCR			COBAS Amplicor HCV v2.0		
	Relapse	Sustained virologic response		Relapse	Sustained virologic response	
Week 4						
Positive	8	4	Relapse/total positive = 8/12 (67%)	6	2	Relapse/total positive = 6/8 (75%)
Negative	1	12	SVR/total negative = 12/13 (92%)	3	14	SVR/total negative = 14/17 (82%)
P-value	0.003			0.02		
End-of-treatment						
Positive	4	0	Relapse/total positive = 4/4 (100%)	0	0	Relapse/total positive = 12/0
Negative	8	16	SVR/total negative = 16/24 (67%)	12	16	SVR/total negative = 16/28 (57%)
P-value	0.01			1.00		

TABLE IV. Characteristics and Quantified Viral Load of HCV RNA in Plasma Which Was HCV RNA Negative by Cobas Amplicor HCV Version 2.0 (Sensitivity, 50 IU/ml)

Patients	Outcome	Sampling	HCV RNA (copies/ml)
1	Relapse	Week 4	3.5
2	Relapse	Week 4	8.0
3	Relapse	EOT	4.9
4	Relapse	EOT	11.0
5	Relapse	EOT	3.5
6	Relapse	EOT	17.3
7	SVR	Week 4	3.5
8	SVR	Week 4	7.6

EOT, End-of-treatment.

Several clinical studies have shown that early viral assessments provide identification of nonresponders to antiviral therapies [Davis et al., 2003; Jensen et al., 2006], and the consensus guidelines of American Association of Study for Liver Disease (AASLD) [Strader et al., 2004] recommended discontinuation of therapy in patients who do not achieve EVR. Moreover, Jensen et al. [2006] suggested that therapy can be discontinued by 24 weeks in patients who achieve rapid virologic response at week 4, because there was no significant difference in rate of sustained virologic response between patients treated for 24 and 48 weeks. However, virologic relapse occurs at various rates among patients with rapid virologic response (9–27%) [Jensen et al., 2006].

The ratios of sustained virologic response among patients who were HCV negative at week 4 of treatment by the present assay and COBAS Amplicor were 12/13 (92%) and 14/17 (82%), respectively, suggesting that undetectability of HCV RNA at week 4 by the more sensitive assay is more predictive of sustained virologic response. The threshold of plasma viral levels in patients with a strong virologic suppression during the early phase that leads to sustained virologic response may be much below 50 IU/ml. The qualitative transcription-mediated amplification assay, which has a higher sensitivity than COBAS Amplicor (detection limit 25–50 copies/ml), showed a slightly higher detection rate for HCV RNA than COBAS Amplicor (18/21 (86%) by transcription-mediated amplification assay versus 16/21 (76%) by COBAS Amplicor) in the testing of week-4 samples from relapsers [Desombere et al., 2005]. Because the sensitivity of the present RT-nested PCR assay is higher than that of the transcription-mediated amplification assay (5 copies/ml vs. 25–50 copies/ml at 95% detection), the predictability of sustained virologic response by the RT-nested PCR assay may be higher than by the transcription-mediated amplification assay.

Despite of the high sensitivity in detecting a single copy, one of nine samples at week four in relapsers was HCV-negative by the present assay. There are two possible explanations for this result. First, antiviral treatment leads to a complete replication arrest in blood, but small quantities of HCV RNA may persist in the liver [Radkowski et al., 2005]. Second, macrophages and lymphocytes may serve as a replication sites for HCV RNA, resulting in failure of detection of HCV RNA in plasma samples [Laskus et al., 2000; Watkins-Riedel et al., 2004; Radkowski et al., 2005]. On the other hand, detection of HCV RNA at week 4 is not well correlated with virologic relapse; 4 of 16 (25%) and 2 of 16 (13%) sustained virologic responders were HCV positive at week 4 by the present assay and COBAS Amplicor, respectively. It may be too early to identify patients with relapse or nonresponse by the detection of HCV RNA at week 4, because an early decision to discontinue treatment at week 4 would be a serious error in patients with the potential for a sustained virologic response.

In testing end-of-treatment samples, the RT-nested PCR assay detected residual HCV RNA in 4 of 12 (33%) relapsers, and 0 of 16 (0%) sustained virologic responders ($P = 0.02$), all of whom were HCV RNA negative by COBAS Amplicor. Despite of the small number of samples, this result suggests that patients who are HCV-positive by the RT-nested PCR assay at the end-of-treatment will relapse with quite a high probability after treatment. The detection rate of 33% in end-of-treatment samples from relapsing patients by the RT-nested PCR assay is similar to that obtained with the transcription-mediated amplification assay [Sarrazin et al., 2001; Desombere et al., 2005]. By retesting end-of-treatment samples with both the RT-nested PCR assay and the transcription-mediated amplification assay, it was found that the more highly sensitive assays can detect residual HCV RNA at the end-of-treatment from relapsers more frequently, and is suggested that virologic relapse occurs on the basis of replication of remaining HCV virions from a very low level after discontinuation of the antiviral therapy.

As previously reported, the level from which strongly suppressed HCV RNA can lead to virologic relapse is thought to be below 50 IU/ml, which may cause errors in the assessment of the efficacy of antiviral therapies [Sarrazin et al., 2001]. Indeed, the transcription-mediated amplification assay detected residual HCV RNA in the Amplicor-negative plasma from relapsers [Morishima et al., 2006]. However, there has been no report to quantify the minimum level of residual HCV RNA before virologic relapse. This study showed that the quantified HCV RNA concentration of relapsers was in the range of 3.5–17.3 copies/ml (Table IV), which is below the detection limit of the transcription-mediated amplification assay as well as that of the COBAS Amplicor HCV assay.

The assay had several limitations in its data and procedures. First, this study enrolled a limited number of patients. Particularly, the number of week-4 samples from relapsers ($n = 9$) was too small to evaluate the statistical difference in the detection rates between the present RT-nested PCR assay and the COBAS Amplicor HCV assay. Second, in comparison with commercially available kits, RT-nested PCR is labor-intensive, and thus is difficult to use for a large number of samples simultaneously. Therefore, the present assay may not be

suitable for routine use in clinical trials. However, since the threshold level of residual HCV RNA capable of producing a virologic relapse is quite low, the present assay may be useful for distinguishing virologic relapse from sustained virologic response.

In conclusion, a highly sensitive assay, almost capable of detecting a single copy of HCV RNA, was developed for the accurate prediction of the efficacy of antiviral therapy in HCV infection. It has been shown that sustained virologic response can be predicted by the undetectablity of HCV RNA at week 4, and, more strongly, that relapse is associated with detection of HCV RNA at the end-of-treatment.

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Results of Immunotherapy for Patients with Unexplained Primary Recurrent Abortions – Prospective Non-Randomized Cohort Study

Taro Nonaka, Koichi Takakuwa, Izumi Ooki, Mami Akashi, Tomokazu Yokoo, Akira Kikuchi, Kenichi Tanaka

Department of Obstetrics and Gynecology, Niigata University School of Medicine, Asahimachi-dori, Niigata, Japan

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Correspondence
Koichi Takakuwa, Department of Obstetrics and Gynecology, Niigata University School of Medicine, 1-757, Asahimachi-dori, Niigata, 951-8510, Japan.
E-mail: obgy@med.niigata-u.ac.jp

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Problem
The present study was conducted to examine the efficacy of immunotherapy for unexplained primary recurrent aborters using paternal lymphocytes.

Method of study
Two hundred and twenty-eight recurrent aborters were prospectively followed up regarding immunotherapy. Of the 228 patients, 165 underwent immunotherapy using freshly prepared paternal lymphocytes and pregnancy outcome was analyzed. No mixed lymphocyte culture reaction-blocking antibodies (MLR-BAbs) were observed in these patients prior to vaccinations. Pregnancy outcome was also analyzed in such as those patients positive for MLR-BAbs and who did not undergo immunotherapy, and in patients negative for MLR-BAbs and who had become pregnant without immunotherapy.

Results
Of the 140 newly pregnant patients after immunotherapy, the pregnancy continued successfully in 110 (78.6%), and the pregnancy continued successfully in 24 of 32 patients (75.0%) who were positive for MLR-BAbs. The success rate of pregnancy was 30.0% in 18 non-immunized patients. Thus, the success rate was significantly higher among patients with immunotherapy and patients positive for MLR-BAbs than in non-immunized patients, negative for MLR-BAbs.

Conclusion
Immunotherapy using paternal lymphocytes is considered to be effective for unexplained primary recurrent aborters negative for MLR-BAbs.

Introduction

Although the etiology of recurrent spontaneous abortion, defined as three or more consecutive early pregnancy losses, is often unclear, several investigators have reported the occurrence of immunologically explainable recurrent spontaneous abortions. Immunotherapy for these patients using the husband's or a third party's leukocytes has been reported for the past quarter of a century.[1–7] The efficacy of this modality, however, is controversial, even among studies with randomized controlled trial.[8–11] This is mainly because of the selection criteria that were adopted, or of the procedure of the immunotherapy. In this study, we show the results of immunotherapy for unexplained primary recurrent aborters using freshly prepared lymphocytes from the husband and attempt to examine the efficacy of the therapy, especially in patients negative for blocking antibodies evaluated by a mixed lymphocyte culture reaction between spouses (MLR-BAbs).

Materials and methods

Patients

A total of 228 primary recurrent aborters, presenting between January 1983 and December 2005, took part in the study. All had provided informed consent. Each participant cohabiting with a single partner had experienced three or more consecutive, confirmed first trimester (i.e. before 14 weeks of gestation) spontaneous abortions. All had experienced no other pregnancy, and so were diagnosed as primary recurrent aborters. None of the participants had indication for presence of any genetic impairment, mullerian anomaly, hormonal deficiency, infectious disease, metabolic disorder, or autoimmune abnomalities, such as antiphospholipid antibodies or lupus anticoagulant disorder, in the course of our systemic work-up.

The patients were initially divided into two groups according to the presence or absence of mixed lymphocyte culture reaction – blocking antibodies (MLR-BAbs) in the sera collected at the time of or after the last abortion. Those with MLR-BAbs were excluded from the immunotherapy, and the natural course of their pregnancy was observed. Sufficient information concerning immunotherapy for recurrent abortion using paternal lymphocytes was given to the patients who were negative for MLR-BAbs, and the immunotherapy was applied only to those patients who requested it. With respect to the patients who did not desire the immune-therapy, the natural course of their pregnancy was observed. If the pregnancy resulted in repeated abortion, the immunotherapy was given at the patient's request.

Thus, the patients were ultimately divided into three groups, that is, those who underwent immunotherapy (group I), those to whom immunotherapy was not given on account of the presence of MLR-BAbs (group II), and those who did not receive immunotherapy at their own request despite being negative for MLR-BAbs (group III). All patients were offered the same degree of care during their pregnancy.

The period of following up the patients, especially those who had not got pregnant, was about 5 years. A patient was deemed to have not gotten pregnant, if the patient concerned had not become pregnant for about 5 years. Concerning the patients who enrolled in this study after January, 2002, the outcome of pregnancy was determined in December, 2006.

Vaccinations Using the Husband's Lymphocytes

The vaccination procedure has been described in detail elsewhere.[3,4,12] Lymphocytes from 100 mL of heparinized peripheral blood of the husband concerned of each patient, in the experimental group, irradiated with 30 Greys of X-rays to prevent any graft-versus-host (GVH) reaction, were suspended in approximately 1 mL of normal physiological saline solution. Each such cell suspension was i.d. injected into the corresponding patient in the experimental group I, immediately after its preparation. Once MLR-BAbs appeared in the sera following a series of vaccinations, the patients were allowed to become pregnant. In our earlier study, the MLR-BAbs were examined after each vaccination.[3] In a recent study, however, the patients underwent two vaccinations 1 month apart, and then given a third vaccination if the MLR-BAbs were not still detected.[4] If the MLR-BAbs could not be detected after the third vaccination, the patients were allowed to become pregnant, and an additional vaccination was given early in the pregnancy.

Mixed Lymphocyte Culture Reaction-Blocking Assay

The blocking effect of sera was investigated in a one-way MLR between spouses. Lymphocytes were collected from heparinized blood via Ficoll-Hypaque gradient centrifugation. Mixed culturing of mitomycin C-treated stimulator cells of the husband and responder cells of the patient was performed for 6 days in a microtiter plate in RPMI 1640 medium containing either pooled human AB serum or test serum. The cultured cells were harvested onto a glass fiber filter after 18 hr of pulsing with ^{3}H-thymidine. DNA synthesis was evaluated by liquid scintillation counting, and the blocking effect (BE) was calculated with the formula

$$BE = (1 - \text{mean cpm of culture in tested serum} / \text{mean cpm of culture in AB serum}) \times 100(\%)$$

A 22% or more MLR-blocking effect was determined as significant, and designated a positive reaction for MLR-BAbs, as reported.[3,4,12]

The procedure used for immunization and the method used for testing MLR-BAbs have been

validated, as one of the authors of this paper has been directly engaged in the immunization and MLR-BAbs test throughout this study.

Statistical Analysis

A non-paired *t*-test was used to analyze whether a significant difference exists among the mean age or the mean number of spontaneous abortions in experimental groups I, II, and III. A chi-squared analysis with Yates' correction or Fisher's exact probability test was used to analyze the probability that pregnancy outcome differed among groups I, II, and III.

Results

The patients accrued in this study are shown in Fig. 1. Of 228 patients, 179 (78.5%) were found to be negative for MLR-BAbs with the MLR-blocking assay, using sera collected at the time of or just after their last abortion. Immunotherapy was given to 156 patients who were negative for MLR-BAbs, at their request. The remaining 23 patients had not opted for immunotherapy, and 18 patients experienced 20 pregnancies without immunotherapy (group III) (pregnancy rate: 78.3%). Of these cases, six pregnancies resulted in normal term delivery, and the remaining 14 pregnancies resulted in repeated spontaneous abortion (Success rate; 30.0%). Nine patients in these unsuccessful cases desired immunotherapy at a subsequent stage. Thus, immunotherapy using paternal lymphocytes was given to 165 patients (group I). Of the 49 patients who were positive for MLR-BAbs, 32 have so far experienced further pregnancy later (group II) (pregnancy rate; 65.3%).

The mean patient age and mean number of abortions did not differ significantly among these three groups (Table I).

For 25 of the 32 patients (78.1%) of group II, the pregnancy culminated in delivery. Of these 25, 21 gave birth to mature infants. One light-for-date infant (an infant whose body weight was less than the 10th percentile of the distribution of the general population (neonates) in Japan[13]) was born in the 38th week of gestation with no anomalies, and one infant was born premature in the 31st week of gestation. Three patients had infants with a major anomaly: one of these infants died just before delivery due to severe omphalocele, one was saved by surgery for intestinal atresia, the other was saved by surgery for meconium peritonitis. Pregnancy had resulted in repeated spontaneous abortion in seven cases. Thus, the success rate in this group was 75.0% (24 of 32).

As mentioned above, 165 patients had undergone vaccination with their husbands' lymphocytes. MLR-BAbs were detected after one or two vaccinations in 148 of these, and after the third vaccination in 14 more patients. Thus, the MLR-BAbs were found in 98.2% of vaccinated patients. In the remaining three patients, no MLR-BAbs were detected even after the third vaccination, and an additional vaccination was given early in the pregnancy.

Of 165 patients, 140 experienced new pregnancies (pregnancy rate; 84.8%), and 110 had their pregnancy continue successfully (success rate: 78.6%). All of these 110 patients have already experienced delivery, and 101 delivered normal mature infants in the 36th week of gestation or later. Four light-for-date infants were born at 36 weeks of gestation or later with no anomaly, and four infants were born as premature delivery in the 28th, 32nd, 33rd, and 34th week of gestation. A major fetal anomaly was observed in one infant diagnosed as having Delange syndrome, who had survived after delivery. Pregnancy resulted in repeated spontaneous abortion in the remaining 28 cases, and in ectopic pregnancy in two cases.

The outcome of pregnancy in groups I, II, and III is shown in Table II. The rate of success was significantly higher in group I and II than group III (78.6% *versus* 30.0%, $P < 0.000001$, 75.0% *versus* 30.0%, $P < 0.001$, respectively).

The outcome of pregnancy in group I according to the number of vaccination(s) necessary to test positive for MLR-BAbs is shown in Table III. The rate of successful pregnancy among the patients in whom the MLR-BAbs appeared after one or two vaccination(s) was 76.0% (96 of 125 cases). In the patients in whom the MLR-BAbs showed up after three vaccinations was 91.7% (11 of 12 cases). The rate was 100% in the group of patients in whom MLR-BAbs could not be observed after three vaccinations and an additional vaccination was applied at an early stage of pregnancy (three of three cases). The success rate did not differ significantly among these three groups.

Discussion

The outcome of immunotherapy using the husband's lymphocytes for unexplained primary recurrent