Table 1. Number of subjects with gross monkeypox-associated lesions confirmed by positive monkeypox virus antigen in each organ, as determined by immunohistochemical analyses

Organ	No. subjects with monkeypox virus antigen in each organ			
	Zr-599/SC	Zr-599/IN	Liberia/SC	Liberia/IN
Total *n*	4	2	3	2
Respiratory				
Trachea	3	0	0	1
Lung	3	0	0	2
Cardiovascular				
Heart	0	0	0	0
Gastrointestinal tract				
Liver	4	1	1	0
Pancreas	3	1	0	1
Oesophagus	0	0	0	0
Stomach	3	0	0	0
Ileum	2	1	0	0
Colon	1	0	0	0
Rectum	4	1	0	0
Endocrine system				
Thyroid	2	0	0	0
Adrenal gland	1	0	0	0
Lymphoreticular system				
Radial lymph node	4	1	1	1
Submandibular lymph node	4	2	1	2
Inguinal lymph node	4	1	2	1
Axillar lymph node	4	1	2	2
Tonsil	4	1	2	2
Thymus	4	1	2	2
Spleen	3	1	0	2
Pharyngeal	4	1	1	1
Genito-urinary tract				
Kidney	0	0	0	0
Bladder	1	0	0	0
Prostate/uterus	2	0	1	1
Testis/ovary	3	1	0	0
Skin				
Skin lesions	4	2	3	2
CNS				
Basal ganglia, lateral lobe, frontal lobe, thalamus or spine	0	0	0	0

20.7 (Table 2). The mean point of the Zr-599/IN group, 15.5, was also higher than that of the Liberia/IN group, 11.5.

The pathology of experimental monkeypox virus infections in non-human primates infected with isolate V79-I-005, which was originally obtained from a fatally infected human from Zaire in 1979, through the respiratory route was reported previously (Zaucha *et al.*, 2001). However, the pathology in monkeys infected with monkeypox virus West African strain has not been studied. Monkeypox-associated gross lesions were demonstrated in the following organs: respiratory system, skin, oral cavity, gastrointestinal tract and lymphoid systems. The pathological findings observed in monkeys infected with the Zr-599 strain were similar to those observed in the previous study (Zaucha *et al.*, 2001). The value of the present study is that the pathology of both Congo Basin and West African monkeypox viruses has been investigated. Differences were observed in fatality rate, severity of monkeypox-associated symptoms, virus genome level and the organs affected. Zr-599 replicated in skin, lymphoid and reticuloendothelial systems, genito-urinary tract organs, respiratory tract organs and gastrointestinal tract organs, whereas Liberia replicated only in the skin, lymphoid and reticuloendothelial systems of the monkeys (Table 1). Whilst the lung of a Zr-599/SC-infected monkey was entirely and diffusely affected by the infection (Supplementary Fig. S2), the lung of a Liberia/SC-infected monkey was histopathologically intact (data not shown). Haemorrhagic diarrhoea was observed only in the Zr-599-infected monkeys. These results suggest that the respiratory and gastrointestinal functions were more severely impaired

Table 2. Difference in virulence between the Zr-599 and Liberia strains of monkeypox virus in non-human primates, as determined by the proposed scoring system for analysis of monkeypox severity

Abbreviations: UDL, under detection level; WBC, white blood cells.

Item	No. animals with score	Mean score in each group			
		Zr-599/SC	Zr-599/IN	Liberia/SC	Liberia/IN
Decrease in body mass (%)	<3, 0; 3–<8, 1; 8–<13, 2; >13, 3	2.3±0.5	1.5±0.7	2.3±1.2	2.0±0.0
Recovery signs in body mass	Positive, 0; negative, 3	2.3±1.5	0	1.0±1.7	0
Duration of decreased activity (days)	None, 0; 1–5, 1; 6–10, 2; >10, 3	2.3±1.0	1.5±0.7	2.0±0.0	1.5±0.7
Duration of decreased meal consumption (days)	None, 0; 1–5, 1; 6–10, 2; >10, 3	2.5±0.6	2.0±1.4	2.3±0.6	1.0±0.0
Fever >1 °C	Negative, 0; positive, 1	0.3±0.5	0	0.3±0.6	0
Drop in body temperature >1.5 °C	Negative, 0; positive, 3	2.3±0.5	0	1.0±1.7	0
Faecal appearance	Normal, 0; watery diarrhoea, 1; haemorrhagic diarrhoea, 3	1.8±1.5	1.5±2.1	0.7±0.6	1.0±0.0
Papulovesicular lesions (no.)	None, 0; 1–50, 1; 51–499, 2; >500, 3	2.3±0.5	1.5±0.7	2.0±1.0	1.0±0.0
Outcome	Non-fatal, 0; fatal, 6	4.5±3.0	0	2.0±3.5	0
Maximum virus genome level [$\log_{10}$(copies ml^{-1})]	UDL, 0; <5, 1; 5–7, 2; >7, 3	2.8±0.5	2.5±0.7	2.3±0.6	1.5±0.7
Virus genome level when sacrificed [$\log_{10}$(copies ml^{-1})]	UDL, 0; <5, 1; 5–7, 2; >7, 3	2.0±1.4	1.0±1.4	1.0±1.0	0.5±0.7
Peripheral WBC count <5000 μl^{-1}	Positive, 0; negative, 1	0.5±0.6	1.0±0.0	1.0±1.0	0.5±0.7
Thrombocytopenia <20 000 μl^{-1}	Positive, 0; negative, 1	0.8±0.5	0.5±0.7	0.3±0.5	1.0±0.0
Anaemia with decrease in haemoglobin level >1.5 g dl^{-1}]	Positive, 0; negative, 1	1.0±0.0	1.0±0.0	1.0±0.0	1.0±0.0
Maximum CRP level [mg dl^{-1}]	<1, 0; 1–<5, 1; 5–<10, 2; >10, 3	2.8±0.5	1.5±0.7	2.3±0.6	1.0±1.4
Mean		30.0±10.4	15.5±9.2	20.7±9.1	11.5±3.5

in the Zr-599-infected monkeys than in the Liberia-infected monkeys.

The difference in the level and course of virus genome detection was consistent with the difference in the pathological findings. Zr-599 replicated more efficiently in the internal organs than did Liberia (Fig. 1; Table 1). The higher level of virus genome detected in the later phase of infection in the Zr-599/SC monkeys might be due to the more efficient replication of Zr-599, particularly in the generalized lymphoid and reticuloendothelial systems, skin, genito-urinary tract organs, respiratory organs and gastrointestinal organs, than that of Liberia in these organs. This feature of Zr-599 might lead to multi-organ failure with malfunctions of respiratory, gastrointestinal and genito-urinary tract organs, resulting in stronger virulence of Zr-599 than of Liberia in non-human primates.

It is believed that Congo Basin strains are more virulent than West African strains in humans, based on clinical studies (Breman *et al.*, 1980; Foster *et al.*, 1972; Ladnyj *et al.*, 1972). Recently, it was reported that a Congo Basin strain, Zr79, which was isolated from a fatal case of monkeypox in Zaire in 1979, was more virulent than the West African strain US03, which was isolated from a non-fatal case of monkeypox in the USA in 2003, using a ground squirrel model for monkeypox virus infection (Sbrana *et al.*, 2007). The clinical course and virological and pathophysiological features of monkeypox virus infections in non-human primates obtained in this study were different from those reported in the ground squirrel model (Sbrana *et al.*, 2007; Tesh *et al.*, 2004). Monkeypox virus infections in non-human primates resemble human monkeypox in terms of pathophysiological profile, making the present study of particular value.

The genetic and molecular mechanism(s) underlying the differences in pathogenesis between Congo Basin and West African strains should be clarified through further studies. It was reported that *D10L*, *D14L*, *B10R*, *B14R* and *B19R* were possibly responsible genes, with *D14L*, an orthologue of vaccina complement protein, as a leading candidate and with *D10L* and *B19R* as less likely candidates (Chen *et al.*, 2005). Although the data are not shown here, the *D14L* gene was confirmed to be absent in the Liberia strain, as in the case of an West African strain, SL-70 (Chen *et al.*, 2005).

In conclusion, it was demonstrated that Zr-599, a Congo Basin strain, was more virulent than Liberia, a West African strain. The difference in virulence might be due to the difference in the sites of virus replication resulting in organ dysfunction: Zr-599 replicated in skin, lymphoid and reticuloendothelial systems, genito-urinary tract organs, respiratory organs and gastrointestinal organs, whereas Liberia replicated only in skin, lymphoid and reticulo-endothelial systems.

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Identification of a site-specific tyrosine recombinase that mediates promoter inversions of phase-variable *mpl* lipoprotein genes in *Mycoplasma penetrans*

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Mycoplasma penetrans has the ability to change its surface lipoprotein profiles frequently. The P35 family lipoproteins encoded by the *mpl* genes are key players in this profile variation. The *M. penetrans* HF-2 genome has 38 *mpl* genes that form three gene clusters. Most of these *mpl* genes have an invertible promoter sequence that is responsible for the ON/OFF switching of individual *mpl* gene expression. Here, we identified the recombinase that catalyses inversions of the *mpl* gene promoters. We focused on two open reading frames of the *M. penetrans* HF-2 genome, namely MYPE2900 and MYPE8180, which show significant homology to the tyrosine site-specific recombinase (Tsr) family proteins. Since genetic tools for *M. penetrans* are still not developed, we cloned the MYPE2900 and MYPE8180 genes and expressed them in *Mycoplasma pneumoniae* and *Escherichia coli*. The promoter regions of the *mpl* genes [*p35* (MYPE6810) or *p42* (MYPE6630) genes] were also introduced into *M. pneumoniae* and *E. coli* cells expressing MYPE2900 or MYPE8180. Inversion of these promoters occurred in the presence of the MYPE2900 gene but not in the presence of the MYPE8180 gene, indicating that the MYPE2900 gene product is the recombinase that catalyses *mpl* gene promoter inversions. We used a PCR-based method to detect *mpl* promoter inversion. This method also enabled us to detect inversions of 10 *mpl* gene promoters in *M. penetrans* HF-2 cells. All these promoter inversions occurred at the 12 bp inverted repeat (IR) sequences flanking the promoter sequence. The consensus sequence of these IRs was proposed as TAAYNNNDATTA (Y=C or T; D=A, G or T).

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INTRODUCTION

Mycoplasmas belong to a group of bacteria with no cell wall and have the minimum range of genome sizes that are necessary for self-replication. Their small genomes (580–1350 kb) sequenced to date lack numerous genes required for biosynthetic pathways, thus reflecting their parasitic lifestyle with a dependence on host organisms for nutrient acquisition. Mycoplasmas usually inhabit the mucosal tissues of specific host organisms. Almost 200 mycoplasma species have been isolated from a wide range of host organisms, including humans. Several of these species are well recognized as pathogens (Sasaki, 2006; Waites *et al.*, 2005). As parasitic bacteria, mycoplasmas can continue to colonize their host even in the presence of specific immune responses. The molecular mechanisms responsible for this immune evasion are not fully understood; however, a number of recent studies have suggested probable mechanisms for continuous infection of mycoplasmas; these include molecular mimicry of host cell components (Jacobs *et al.*, 1995), modulation of host immunity by mycoplasmal cell components (Rottem, 2003), invasion of host cells (Baseman *et al.*, 1996) and generation of surface antigen variants of mycoplasmas (Denison *et al.*, 2005; Rosengarten *et al.*, 2000). Among these strategies, surface antigenic variation is a commonly observed phenomenon in many mycoplasma species (Citti *et al.*, 2005; Yogev *et al.*, 2002). Surface variations may play important roles in interaction between mycoplasmas and host cells during

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Abbreviations: Tsr, tyrosine site-specific recombinase; Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sp, spectinomycin; IR, inverted repeat.

Two supplementary tables, listing the oligonucleotides used in this study, are available with the online version of this paper.

infection. In most of the known cases, the variable surface molecules of mycoplasmas are lipoproteins. Depending upon the species, these lipoproteins are encoded by single or multiple genes and undergo frequent phase and size variation during mycoplasma growth (Citti *et al.*, 2005). Various genetic mechanisms are used to modulate the expression of these lipoprotein genes. These mechanisms include DNA rearrangements (Glew *et al.*, 2002; Shen *et al.*, 2000), nucleotide insertions and deletions in the gene regions (Glew *et al.*, 1998; Yogev *et al.*, 1991), gene conversions (Noormohammadi *et al.*, 2000), and site-specific DNA recombinations (Chopra-Dewasthaly *et al.*, 2008; Lysnyansky *et al.*, 2001). Characterization of these genetic mechanisms may provide us with not only a detailed understanding of the surface antigenic variation mechanism of mycoplasmas but also an insight into the survival strategy of minimalist bacteria through utilization of their small genomes.

Mycoplasma penetrans was first isolated from a urine sample of a human immunodeficiency virus (HIV)-infected patient (Lo *et al.*, 1992). It was also isolated as a potential aetiological agent from a primary antiphospholipid syndrome patient without HIV infection (Yáñez *et al.*, 1999). Although the pathogenicity of *M. penetrans* to humans remains questionable, the characteristics of this bacterium (i.e. invasion of eukaryotic cells, toxicity to chick embryo, and haemolytic and haemoxidative activity) (Girón *et al.*, 1996; Kannan & Baseman, 2000; Lo *et al.*, 1993) suggest its potential pathogenicity to humans. *M. penetrans* also has the ability to change its surface antigenicity frequently by changing the expression pattern of the P35 family lipoproteins (Neyrolles *et al.*, 1999; Röske *et al.*, 2001). The P35 family lipoproteins are encoded by the *mpl* genes and, thus far, 38 *mpl* genes have been found in the *M. penetrans* HF-2 genome. Of these 38 genes, 30 form a large gene cluster in a 50 kb region of the genome (Horino *et al.*, 2003; Sasaki *et al.*, 2002). *M. penetrans* uses a unique mechanism to modulate the expression of these *mpl* genes. Most of the *mpl* genes have an independent promoter for their expression. These promoter sequences are present in approximately 135 bp DNA regions and are flanked by 12 bp inverted repeats (IRs). Inversion of these promoters causes ON/OFF switching of individual *mpl* genes. The invertible promoter elements also contain unique sequences that form a terminator-like structure depending on the direction of promoters (see Fig. 4a). This terminator-like structure may serve to prevent readthrough transcription from preceding genes or antisense transcription from OFF configuration promoters (Horino *et al.*, 2003). Thus, the *mpl* genes have unique genetic switches, which possess promoter and terminator functions in the short DNA region. To further characterize these unique genetic switches and understand the nature of antigenic variation of *M. penetrans*, in this study we attempted to identify the factors involved in *mpl* gene promoter inversions. For this purpose, we reconstructed the *mpl* promoter inversion system in *Mycoplasma pneumoniae* and *Escherichia coli* cells.

METHODS

Bacterial strains and culture conditions. *M. penetrans* strain HF-2 (Yáñez *et al.*, 1999) and *M. pneumoniae* strain M129 (Lipman *et al.*, 1969) were cultured in PPLO medium at 37 °C as described previously (Horino *et al.*, 2003; Kenri *et al.*, 2004). Transformation of *M. pneumoniae* M129 with the staphylococcal transposon Tn*4001*mod vectors was performed by the electroporation method (Hedreyda *et al.*, 1993). *M. pneumoniae* transformants were selected in PPLO liquid medium containing 18 μg gentamicin (Gm) ml^{-1} or 15 μg chloramphenicol (Cm) ml^{-1}. Genomic DNAs of the mycoplasma strains were extracted by the QIAamp mini kit (Qiagen) and were used as templates for PCR to construct the plasmids. *E. coli* strains were grown in Luria–Bertani (LB) medium with or without 50 μg ml^{-1} of ampicillin (Ap), kanamycin (Km) or spectinomycin (Sp), or 15 μg Cm ml^{-1} to select the plasmid markers.

Cloning of the *p42* and *p35* promoter regions. PCR primers used for cloning are listed in Supplementary Table S1 (available with the online version of this paper). An approximately 3.2 kb region containing the *p42 mpl* gene (MYPE6630) and its invertible promoter region (see Figs 1a and 5a) was amplified from *M. penetrans* HF-2 genomic DNA by PCR with primers p42-F and p42-R. To minimize mutations caused by PCR amplification, a high-fidelity DNA polymerase (PyroBest; TaKaRa) was used. The corresponding region of the *p35 mpl* gene (MYPE6810) was also amplified from *M. penetrans* HF-2 by PCR with primers p35-F and p35-R. The amplified *p42* and *p35* regions were inserted into the pENTR/D-TOPO plasmid by using the TOPO cloning system according to the manufacturer's instructions (Invitrogen), resulting in plasmids pAH501 and pAH502 (Table 1). The cloned *p42* and *p35* promoter sequences on these plasmids were in the ON direction. To introduce the *p42* promoter sequence into *M. pneumoniae* cells, the cloned *p42* region on plasmid pAH501 was transferred to the *Sma*I site of the Tn*4001*mod vector plasmid pISM2062.2 (Knudtson & Minion, 1993) by using the Gateway cloning technique (Invitrogen). Briefly, pISM2062.2 was converted into the Gateway destination vector by inserting the Gateway vector conversion system (reading frame cassette A) at the *Sma*I site. The *p42* sequence of the pAH501 plasmid was transferred to the pISM2062.2 destination vector by the LR reaction of the Gateway system. The resulting plasmid was designated pAH511 (Table 1) and introduced into *M. pneumoniae* M129 by electroporation. The *p42* and *p35* promoter sequences on the pAH501 and pAH502 plasmids, respectively, were also transferred to the *Sma*I site of the pCL1920 plasmid, which has a pSC101 replicon (Lerner & Inouye, 1990), by using the Gateway cloning system for introduction into *E. coli* cells. These plasmids were designated pAH521 and pAH522, respectively (Table 1).

Construction of the MYPE2900 and MYPE8180 expression clones. PCR primers used for plasmid construction are listed in Table S1. MYPE2900 was amplified from *M. penetrans* HF-2 genomic DNA by PCR with primers 2900-F-TOPO and 2900-R-Asc. MYPE8180 was also amplified using primers 8180-F-TOPO and 8180-R-Asc. The amplified MYPE2900 and MYPE8180 fragments were inserted into the plasmid pENTR/D-TOPO by using the TOPO cloning system, resulting in plasmids pAH301 and pAH302 (Table 1). The cloned MYPE2900 and MYPE8180 on the pAH301 and pAH302 plasmids were sequenced to confirm that no mutations were incorporated during the PCR cloning. To express MYPE2900 and MYPE8180 in *M. pneumoniae* cells, the *tuf* promoter (P_{tuf}) sequence of *M. pneumoniae* was linked to MYPE2900 and MYPE8180. The P_{tuf} sequence was amplified from *M. pneumoniae* M129 genomic DNA by PCR using primers tuf-F-Bam-TOPO and tuf-R-Afl. The amplified *tuf* promoter fragment was digested with *Bam*HI and *Afl*III and ligated into the *Bam*HI–*Nco*I sites of pAH301 and pAH302, resulting in plasmids pAH303 and pAH304 (Table 1). The MYPE2900 and

Table 1. Plasmids constructed in this study

Plasmid	Vector	Marker	Gene
pAH301	pENTR/D-TOPO	Km^R	MYPE2900
pAH302	pENTR/D-TOPO	Km^R	MYPE8180
pAH303	pENTR/D-TOPO	Km^R	P_{tuf}-MYPE2900
pAH304	pENTR/D-TOPO	Km^R	P_{tuf}-MYPE8180
pAH311	pKV104 (Tn*4001*mod)	Ap^R, Cm^R	P_{tuf}-MYPE2900
pAH312	pKV104 (Tn*4001*mod)	Ap^R, Cm^R	P_{tuf}-MYPE8180
pAH331mut	pColdI	Ap^R	P_{cspA}-MYPE2900mut
pAH332	pColdI	Ap^R	P_{cspA}-MYPE8180
pAH332mut	pColdI	Ap^R	P_{cspA}-MYPE8180mut
pAH501	pENTR/D-TOPO	Km^R	*p42* and promoter region
pAH502	pENTR/D-TOPO	Km^R	*p35* and promoter region
pAH511	pISM2062.2 (Tn*4001*mod)	Ap^R, Gm^R	*p42* and promoter region
pAH521	pCL1920 (pSC101 replicon)	Sp^R	*p42* and promoter region
pAH522	pCL1920 (pSC101 replicon)	Sp^R	*p35* and promoter region

MYPE8180 genes on the pAH303 and pAH304 (P_{tuf}-MYPE2900 and P_{tuf}-MYPE8180, respectively) were then transferred to the *Sma*I site of the Tn*4001*mod vector plasmid pKV104 (Hahn *et al.*, 1999) by using the Gateway cloning technique. The resulting Tn*4001*mod plasmids pAH311 and pAH312 (Table 1) were used to transform *M. pneumoniae*. To express the MYPE2900 and MYPE8180 genes in *E. coli*, these genes were amplified from *M. penetrans* HF-2 genomic DNA by PCR with the primer pairs 2900-F-SacTGG and 2900-R-Xho or 8180F-Nde and 8180-R-Eco, respectively. The amplified MYPE2900 fragments were digested with *Sac*I and *Xho*I and inserted into the *SacI–XhoI* site of plasmid pColdI (TaKaRa), resulting in plasmid pAH331mut (Table 1). The MYPE2900mut gene on plasmid pAH331mut was sequenced, and the conversion of the codon UGA to UGG was confirmed (nt position 28–30 of the MYPE2900 gene). The amplified MYPE8180 fragments were digested with *Nde*I and *Eco*RI and inserted into the *NdeI–Eco*RI site of plasmid pColdI, resulting in pAH332 (Table 1). Two UGA codons present in MYPE8180 sequences (at nt positions 145–147 and 517–519) were converted to UGG by using the GeneTailor site-directed mutagenesis system according to the manufacturer's instructions (Invitrogen). After mutagenesis, the nucleotide sequence of the MYPE8180mut gene was confirmed by sequencing. The plasmid carrying the MYPE8180mut gene was designated pAH332mut (Table 1). Plasmids pAH331mut and pAH332mut were introduced into *E. coli* BL21(DE3) to express MYPE2900 and MYPE8180.

Protein analysis. *E. coli* BL21(DE3) strains harbouring plasmids pAH331mut, pAH332mut or pColdI were grown at 37 °C until the mid-exponential phase. The cultures were maintained at 15 °C for 30 min, and 0.2 mM final concentration of IPTG was added. After the addition of IPTG, the cultures were maintained at 15 °C with shaking for 24 h. *E. coli* cells were collected from a 1 ml volume of the cultures by centrifugation at 20 000 *g* for 2 min and lysed by adding 150 µl of a sample loading buffer for SDS-PAGE. The samples were then subjected to SDS-PAGE at a load of 15 µl per lane. The proteins were visualized by Coomassie brilliant blue staining. For Western blot analysis, the proteins, separated by SDS-PAGE, were transferred onto a nitrocellulose membrane (Bio-Rad). Monoclonal antibody specific for His_6-tag (Cell Signaling Technology) was used at a 1 : 1000 dilution to detect His_6-tagged MYPE2900 and MYPE8180 proteins. The reacting antibodies were detected with an alkaline phosphatase-conjugated secondary antibody (goat anti-mouse immunoglobulin G; Promega) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) Colour Development Substrate (Promega) according to the manufacturer's instructions.

PCR detection of *mpl* gene promoter inversion. The PCR primer sets designed for the detection of inversions of the *mpl* gene promoters are listed in Supplementary Table S2. Each primer set consists of three primers [promoter (P), forward (F) and reverse (R) primers]. Two PCRs were performed using these primer sets (with FP primers or RP primers) for each of the corresponding *mpl* gene promoters to detect the inversion event (see Figs 1 and 5). Genomic DNAs from *M. penetrans* HF-2 or *M. pneumoniae* M129-*p42* were analysed with this PCR. The *E. coli* plasmid DNAs carrying the *p42* or *p35* promoter regions were also analysed with this method. Genomic DNAs of *M. pneumoniae* M129-*p42* transformants were extracted from 25 ml of culture by the QIAamp mini kit. The genomic DNAs were diluted and 1 ng of each (about 1×10^6 copies of *M. pneumoniae* M129 genome) was examined by PCR. For purification of *M. penetrans* HF-2 genomic DNA, a single colony of *M. penetrans* HF-2 on a PPLO agar plate [derived from the same culture stock used for genome sequencing (Sasaki *et al.*, 2002)] was picked up and cultured in 2 ml PPLO liquid medium at 37 °C. After growth, 100 µl of the culture was added to 25 ml fresh PPLO liquid medium and cultured until the medium colour changed to orange. Genomic DNA was extracted from 1 ml of the culture by the QIAamp mini kit. One nanogram of the purified genomic DNA (about 6×10^5 copies of *M. penetrans* HF-2 genome) was subjected to the PCR analysis. *E. coli* plasmid DNAs were extracted from 4 ml of the *E. coli* cultures by the QIAprep Spin Miniprep kit (Qiagen). One microlitre of plasmid solution was subjected to PCR examination. The PCR mixture *Premix Ex Taq* Hot Start Version (TaKaRa) was used for the PCR examination, and the PCR was performed under the following conditions: 30 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 2.5 min. The PCR products were analysed by 0.8 % (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining. For sequencing analysis, the PCR products were excised from agarose gels and extracted by using the MinElute gel extraction kit (Qiagen). The extracted DNAs were sequenced by the dye-termination method by using corresponding P, F, or R primers and an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

RESULTS

MYPE2900 belongs to the tyrosine site-specific recombinase (Tsr) family and catalyses *p42* promoter inversion in *M. pneumoniae* cells

The gene annotation process for the complete genome sequence of *M. penetrans* HF-2 indicated that two open

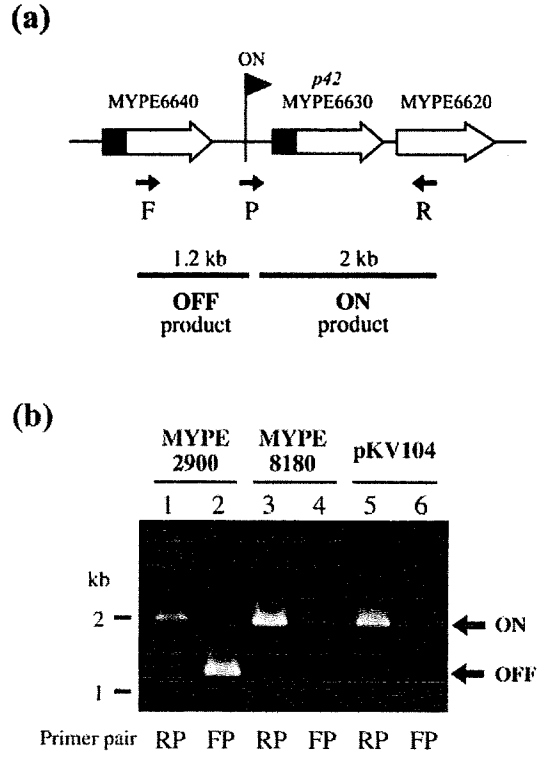


Fig. 1. Promoter inversion of the *p42* (MYPE6630) gene in *M. pneumoniae* cells. (a) The PCR method for detecting promoter inversion. The gene region containing *p42* and its invertible promoter is illustrated schematically. Three arrowed boxes represent the MYPE6640, *p42* and MYPE6620 genes (also see Fig. 5a). The triangle indicates the invertible promoter of the *p42* gene (in the ON direction). Filled boxes in the MYPE6640 and *p42* genes indicate the signal peptide region of lipoproteins. The positions of the three primers, namely, F (forward), R (reverse) and P (promoter), are shown by arrows. The ON and OFF products (approx. 2 and 1.2 kb, respectively) are amplified by two PCRs with primer sets FP or RP, depending on the direction of the *p42* promoter. The primer sequences are listed in Supplementary Table S2. (b) Agarose gel (0.8 %, w/v) electrophoresis pattern of the PCR products. Genomic DNAs of M129-*p42* cells carrying P_{tuf}-MYPE2900 (lanes 1 and 2), P_{tuf}-MYPE8180 (lanes 3 and 4) or pKV104 vector alone (lanes 5 and 6) were analysed by the PCR method. Primer pairs used for the PCR analysis are shown below. Arrows indicate positions of the ON and OFF products.

reading frames, namely MYPE2900 and MYPE8180, show significant homology to the Tsr family genes (Horino *et al.*, 2003; Sasaki *et al.*, 2002). The amino acid sequences of MYPE2900 and MYPE8180 share relatively high homologies to the Tsr of *Mycoplasma pulmonis* and *Mycoplasma agalactiae*, which were previously shown to mediate site-specific DNA inversions (Chopra-Dewasthaly *et al.*, 2008; Sitaraman *et al.*, 2002). We designed experiments to define whether the MYPE2900 or MYPE8180 products can mediate promoter inversion of *mpl* genes. Since genetic tools are still unavailable for *M. penetrans*, an *mpl* promoter inversion system was reconstructed in *M. pneumoniae* cells. For this purpose, a 3.2 kb DNA fragment containing the *p42 mpl* gene and its promoter region of *M. penetrans* HF-2 was introduced into *M. pneumoniae* strain M129 by using the staphylococcal transposon Tn*4001*mod vector, which carries a Gm^R marker (see Methods). Genomic DNA was extracted from the whole transformed population (M129-*p42*; the *p42* promoter sequence is inserted at random in the chromosome) and was analysed by PCR to examine the direction of the *p42* promoter. This PCR analysis (Fig. 1a) gave only the 2 kb product from the genomic DNA of M129-*p42* (data not shown), indicating that the direction of the *p42* promoter in M129-*p42* cells was in the ON direction and that *p42* promoter inversion does not occur in *M. pneumoniae* cells, regardless of the position of the *p42* promoter in the *M. pneumoniae* chromosome.

We then introduced recombinase genes into the M129-*p42* cells. The MYPE2900 and MYPE8180 clones linked with *M. pneumoniae* P_{tuf} promoter were introduced into M129-*p42* cells by using the Tn*4001*mod derivative vector that carries a Cm^R marker (see Methods). Thus transformants of M129-*p42* (Gm^R-Cm^R) having the *p42* promoter sequence and the recombinase genes were obtained. We then extracted genomic DNA from these transformants (whole transformed population) and examined the direction of the *p42* promoter by PCR. This analysis showed that both 2 and 1.2 kb products were amplified from the genomic DNA carrying the MYPE2900 gene (Fig. 1b, lanes 1 and 2). However, only 2 kb product was obtained from the genomic DNAs carrying the MYPE8180 gene (Fig. 1b, lanes 3 and 4) or Tn*4001*mod vector alone (Fig. 1b, lanes 5 and 6). These results suggested that the MYPE2900 protein is the recombinase that has an activity to invert the *p42* promoter sequence.

Reconstruction of the *mpl* promoter inversion system in *E. coli*

To further characterize *mpl* promoter inversion and to develop a simple experimental system, we reconstructed the *mpl* promoter inversion system in *E. coli*. Similar to standard mycoplasmal genes, MYPE2900 and MYPE8180 have UGA codons, which are translated into tryptophan in mycoplasma cells. MYPE2900 has a single UGA codon, and MYPE8180 has two UGA codons. Since these UGA codons block full translation of MYPE2900 and MYPE8180 in *E. coli*, we converted these UGA codons into UGG. The converted MYPE2900 and MYPE8180 genes (MYPE2900mut and MYPE8180mut) were linked with the *E. coli cspA* promoter and His_6-tag sequence at the 5′ end on plasmid pAH331mut and pAH332mut, respectively (see Methods and Table 1). Plasmids pAH331mut and pAH332mut were introduced into *E. coli* BL21(DE3), and the strain was cultured under appropriate conditions to activate the *cspA* promoter (see Methods). After 24 h culture at 15 °C with or without IPTG, the expression of MYPE2900 and MYPE8180 proteins was detected by immunoblot analysis specific for His_6-tag (Fig. 2). The

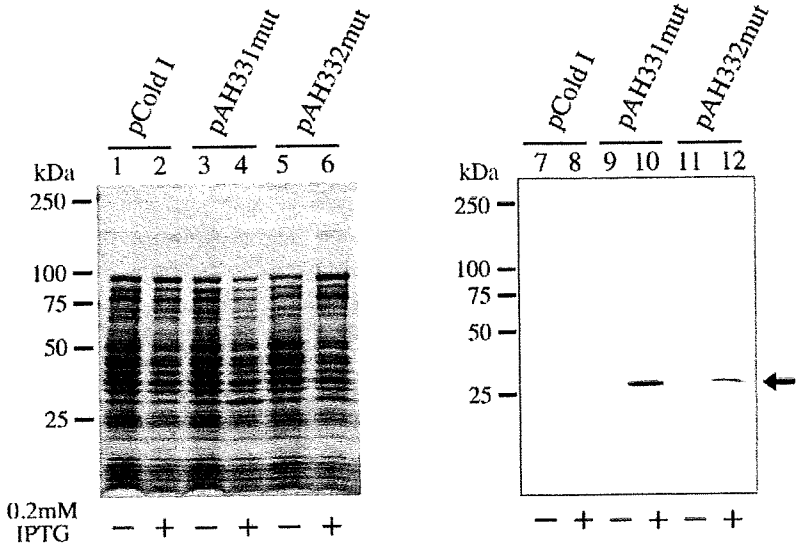


Fig. 2. Production of MYPE2900 and MYPE8180 proteins in *E. coli*. SDS-PAGE gradient gel (5–20%, w/v, lanes 1–6) and immunoblot analysis (lanes 7–12) patterns of proteins from *E. coli* transformants. *E. coli* strains carrying pAH331mut (lanes 3, 4, 9 and 10), pAH332mut (lanes 5, 6, 11 and 12) or pColdI vector plasmids (lanes 1, 2, 7 and 8) were analysed. Addition (+) or no addition (−) of 0.2 mM IPTG during culture is indicated below. His_6-tagged MYPE2900 and MYPE8180 proteins (MYPE2900mut and MYPE8180mut gene products) were detected using a monoclonal antibody against His_6-tags on a blotted nitrocellulose membrane (lanes 7–12). The MYPE2900 and MYPE8180 proteins detected are indicated by an arrow.

MYPE2900 and MYPE8180 proteins were produced apparently in the presence of IPTG (Fig. 2, lanes 10 and 12), although the reason for the low expression level of MYPE8180 compared to MYPE2900 is not clear. In addition, a weak expression of MYPE2900 was observed in the absence of IPTG (Fig. 2, lane 9).

We then constructed pSC101 derivative plasmids carrying the *p42* or *p35 mpl* genes and their promoter regions from the *M. penetrans* HF-2 genome (approx. 3.2 kb DNA region). These plasmids, namely pAH521 and pAH522 (Table 1), were introduced into *E. coli* BL21(DE3) harbouring plasmid pAH321mut or pAH322mut. After culturing these *E. coli* strains under appropriate conditions to activate the *cspA* promoter, the plasmids were recovered from the cells and analysed by PCR to determine the directions of the *p42* and *p35* promoter sequence (see Methods). As shown in Fig. 3, the PCR analysis revealed ON and OFF direction products of the *p42* and *p35* promoter sequences in the presence of the MYPE2900mut gene (Fig. 3, lanes 1, 2, 3, 4, 9, 10, 11 and 12). The absence of 0.2 mM IPTG did not affect this inversion event (Fig. 3, lanes 1, 2, 9 and 10), suggesting that a small amount of MYPE2900 recombinase was sufficient to catalyse *p42* and *p35* promoter inversions. On the other hand, only ON direction products were obtained in the presence of the MYPE8180mut gene (Fig. 3, lanes 5, 7, 13 and 15), indicating that the promoters *p42* and *p35* were locked in the ON direction in these conditions. These results strongly support the conclusion that the MYPE2900 protein is the recombinase that mediates DNA inversions of the *mpl* gene promoters. We also performed the same experiments in *E. coli* strain YK1340 (Wada *et al.*, 1988), which lacks the histone-like protein HU. Even in the YK1340 background, inversion of the *p42* and *p35* promoters occurred only in the presence of the MYPE2900mut gene (data not shown). The recombination sites of DNA inversion (IR sequences) of the *p42* and *p35* promoters are TAACATAAATTA and TAACAAGAATTA, respectively. The MYPE2900 recombinase can probably recognize both these sequences as a recombination site.

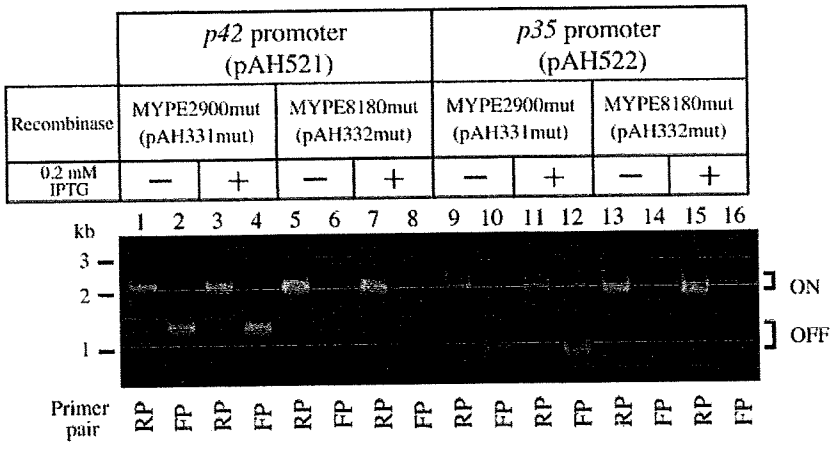
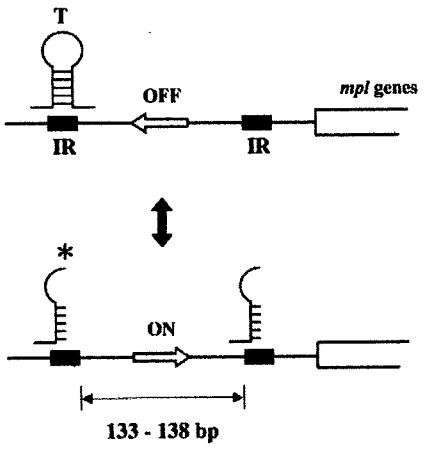


Fig. 3. Electrophoresis patterns of PCR analysis of *p42* and *p35* promoter inversions in *E. coli* cells. The PCR products were electrophoresed on a 0.8% (w/v) agarose gel. PCR analysis results of the *p42* promoter (lanes 1–8) and *p35* promoter (lanes 9–16) are shown. The recombinase genes present in *E. coli* cells (MYPE2900mut or MYPE8180mut) are indicated. Addition (+) or no addition (−) of 0.2 mM IPTG during culture is indicated. Primer sets used for PCR analysis (FP or RP) are indicated below. The positions of PCR products from the ON and OFF direction promoters are indicated on the right.

(a)



(b)

MYPE ORFs	Inverted repeat sequences
6840	T A A T T A G A A T T A
6830	T A A T A T A A A T T A
6820 (*p34A*)	T A A T A G G A A T T
6810 (*p35*)	T A A C A A G A A T T A
6800 (*p30*)	T A A T C A G A A T T A
6780 (*p38*)	T A A T T G G A A T T A
6750	T A A T G T A A A T T A
6740	T A A C A C C A A T T A
6730	T A A C T T G A A T T A
6720	T A A T A A A G A T T A
6710	T A A C G A T A A T T A
6690	T A A C T G G A A T T A
6680	T A A C T G G A A T T A
6670	T A A T G A G A A T T A
6660	T A A C A G G T A T T A
6650	T A A C G C C A A T T A
6640	T A A T A A G A A T T A
6630 (*p42*)	T A A C A T A A A T T A
6580	T A A C G G A A A T T A
6570	T A A T T G G A A T T A
6560	T A A C A C C A A T T A
6550	T A A C T T G A A T T A
6540	T A A C G C T A A T T A
6530	T A A C T G A A A T T A
6520	T A A T A A G A A T T A
6510	T A A T A G G T A T T A
6500	T A A C G A G A A T T A
6490	T A A C A G G A A T T A
2690	T A A T G G G A A T T A
2700	T A A T G C A A A T T A
7400	T A A C A A T A A T T A
consensus	T A A Y N N N D A T T A

Fig. 4. Alignment of IR sequences of the *mpl* gene promoters. (a) Schematic illustration of *mpl* gene promoter region. The IRs (filled boxes) are present at positions separated by 133–138 bp sequences that contain promoter-like sequences (open arrows). The terminator-like sequence (T) is formed upstream of the OFF direction promoter but is disturbed in the ON promoter configuration (asterisk). (b) Twelve-basepair IR sequences found in 30 *mpl* gene promoter regions were aligned. In the IR of the MYPE6820 (*p34A*) gene, one nucleotide mismatch (A:C) is present. The position of mismatch is shaded. The consensus sequence of IRs deduced from the alignment is shown at the bottom (D=A, G or T; N=A, C, G or T; Y=C or T).

Detection of inversions of *mpl* gene promoters in *M. penetrans*

To characterize the consensus sequence of the recombination site of the *mpl* gene promoters, we aligned 30 IR sequences found in the promoter regions of the *mpl* genes in the *M. penetrans* HF-2 genome. The alignment shown in Fig. 4(b) revealed that nucleotides TAA and ATTA at both ends of 12 bp IRs were conserved; however, five nucleotides in the middle of the repeats differed from each other. The consensus sequence was proposed as TAAYNNNDATTA. To determine whether *mpl* promoter inversions actually occur at these various IR sequences in *M. penetrans* cells, we extended the PCR-based inversion detection method. In addition to the primer sets for *p42* and *p35* promoter regions, we designed eight sets of PCR primers for MYPE6840, 6780, 6740, 6690, 6660, 6560, 6530 and 6490 gene promoters (Supplementary Table S2 and Fig. 5a). Of these 10 promoters, the *p42* and *p35* promoters were in the ON direction, and the others were in the OFF direction in the consensus genome sequence data of *M. penetrans* HF-2 (Fig. 5a). To detect the inverted form of these promoters, we analysed *M. penetrans* HF-2 genomic DNA (1 ng template) by the PCR method with 10 sets of primers (Fig. 5a). This PCR analysis showed that amplification products from both the ON and the OFF direction promoters (approx. 2 and 1 kb products) were obtained from all the 10 promoters examined (Fig. 5b), although the amounts of the ON direction products from MYPE6840 and MYPE6660 promoters and the OFF direction product from MYPE6810 (*p35*) were low (Fig. 5b, lanes 1, 4 and 11). We analysed the nucleotide sequences of the PCR products of ON and OFF directions and confirmed that inversion of these promoter sequences occurred at the 12 bp IR sequences listed in Fig. 4(b) (data not shown).

DISCUSSION

In this study, we identified the recombinase that catalyses inversion of the *mpl* gene promoters. Reconstruction of the *mpl* promoter inversion system (recombinase and recombination sites) in *M. pneumoniae* and *E. coli* cells clearly showed that the MYPE2900 gene product has an activity to invert the *p35* and *p42* promoters. These experiments indicated that *mpl* promoter inversion is catalysed by a single factor of *M. penetrans*. In some other site-specific recombination systems, additional factors such as nucleoid-associated proteins are required for DNA recombination together with recombinase (Esposito *et al.*, 2001; Wada *et al.*, 1989). However, these factors may not be essential in *mpl* promoter inversions. The recombination sites (12 bp IR) of the *p42* and *p35* genes are similar but slightly different, suggesting that MYPE2900 recombinase has flexibility in recognizing the recombination site sequences. The *mpl* promoters controlled by MYPE2900 are one of the smallest genetic switches in bacteria (Fig. 4a). MYPE2900 recombinase and its short recombination site may be utilized as a tool for genetic engineering such as modulation of recombinant gene expressions, inversion or excision of specific chromosomal DNA regions, or construction of gene clones by DNA recombination. To develop these tools, the *mpl* inversion systems reconstructed in *E. coli* (with

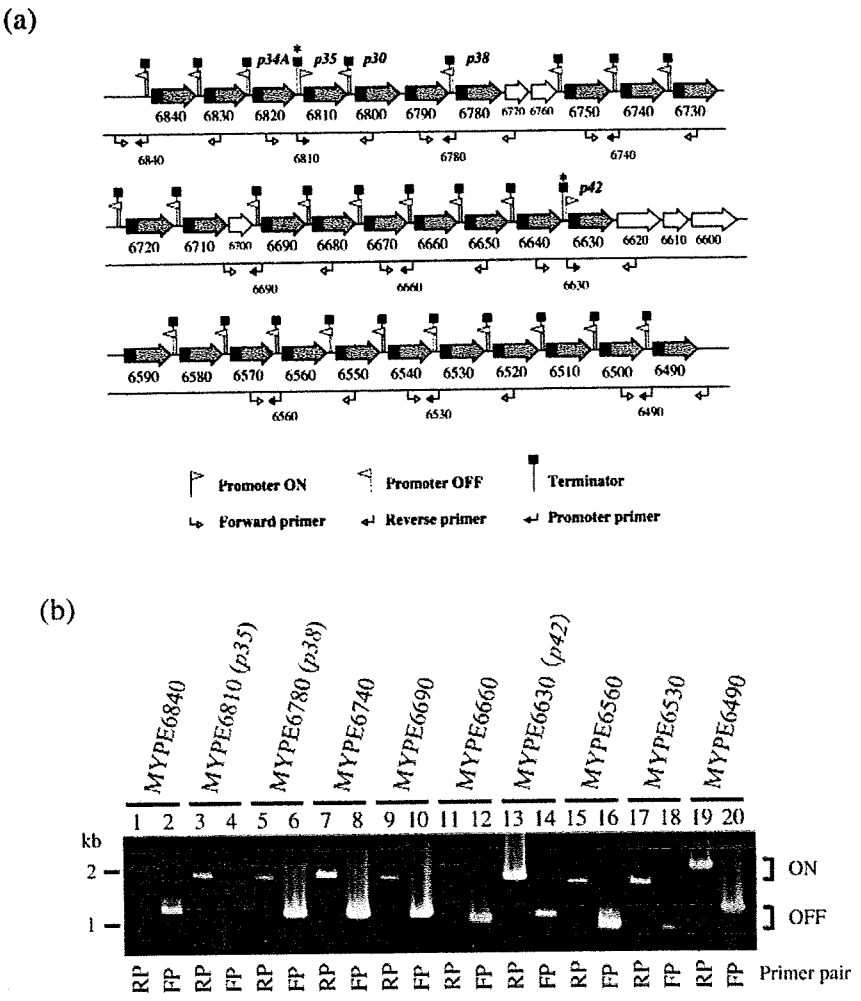


Fig. 5. Detection of promoter inversion of selected *mpl* genes in *M. penetrans* cells by PCR. (a) Schematic illustration of the largest *mpl* gene cluster of the *M. penetrans* HF-2 genome (Horino *et al.*, 2003). The figure was prepared on the basis of the whole genome sequencing data of *M. penetrans* HF-2 (Sasaki *et al.*, 2002; GenBank accession no. BA000026). Grey arrowed boxes represent the *mpl* genes in this region. White arrowed boxes represent the non-*mpl* genes. Filled boxes in the *mpl* genes represent the signal peptide region of these genes. Open triangles indicate invertible promoters and their ON and OFF directions. Squares represent terminator-like sequences. The terminator-like sequences in inactive form are marked with an asterisk (*p35* and *p42* genes, see Fig. 4a). Small arrows below the genes indicate the relative positions of PCR primers used for the detection of promoter inversions. (b) PCR analysis of promoter inversions of 10 *mpl* genes. The PCR products were electrophoresed on a 0.8% (w/v) agarose gel. The promoters analysed are indicated above. P and R primer sets were used to detect the ON direction promoters (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19). P and F primer sets were used to detect the OFF direction promoters (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20). The sequences of each primer set are listed in Supplementary Table S2. The positions of ON and OFF products are indicated on the right.

His_6-tagged MYPE2900) are useful for detailed characterization of the recombination mechanism and designing of genetic tools.

In our previous study, we did not perform direct detection of *mpl* promoter inversions in *M. penetrans* cells (Horino *et al.*, 2003). In the present study, we used a PCR-based method and found the presence of an inverted form of 10 *mpl* gene promoters (including the *p35* and *p42* promoters) in *M. penetrans* genomic DNA. Inversions of these *mpl* gene promoters occurred at the 12 bp IR sequences that are similar to those of the *p35* and *p42* promoters (Fig. 4b), suggesting that all these promoter inversions may be catalysed by MYPE2900 recombinase in *M. penetrans* cells. The 12 bp IR sequences of all *mpl* genes slightly differ from each other and show the consensus sequence TAAYNNNDATTA. This type of dyad motif is commonly present as a recognition site of site-specific DNA recombinases. However, the 12 bp sequence is considerably shorter than that of the other known recombination sites recognized by site-specific DNA recombinases (Glew *et al.*, 2002; Honarvar *et al.*, 2003; Li *et al.*, 2002; Lysnyansky *et al.*, 2001; Sitaraman *et al.*, 2002). The variation in 12 bp IRs of the *mpl* gene promoters may contribute to the prevention of inappropriate recombination between different *mpl* gene promoters and to maintaining the structure of the *mpl* gene cluster (Fig. 5a). The variation in recombination sites may also affect the frequency of inversion of individual promoters. In this study, we did not examine the inversion frequency of each promoter. This could be evaluated by a real-time PCR method or by using reconstruction systems in *M. pneumoniae* and *E. coli* cells in further studies.

Another characteristic feature of the *mpl* promoter inversion system is the location of the recombinase gene. In many site-specific recombination systems, which control antigenic variations, the Tsr family genes tend to be located near the recombination sites (Chopra-Dewasthaly *et al.*, 2008; Komano, 1999; Kuwahara *et al.*, 2004; Ron *et al.*, 2002). However, in the *mpl* promoter inversion system, the MYPE2900 gene is located approximately 500 kb apart from the major *mpl* gene cluster. The other two minor *mpl* gene clusters are also separated by approximately 600 or 40 kb from the MYPE2900 gene (Horino *et al.*, 2003). In addition, MYPE2900 recombinase efficiently catalysed the

inversions of the *p42* and *p35* promoters in a *trans*-acting manner in *E. coli* cells (the MYPE2900 gene and the *p42* or *p35* promoters were present in different plasmids). Probably, there is no *cis*-acting effect between the MYPE2900 gene and *mpl* promoter recombination sites.

The amino acid sequence of MYPE8180 strongly suggests that it is also a Tsr. However, in our experimental conditions, the MYPE8180 product did not show recombinase activity in *p42* and *p35* promoter inversions. The reconstruction systems in this study are not identical to the natural context in *M. penetrans* cells, so that there are still possibilities that MYPE8180 recombinase takes part in the *mpl* system with additional factors in *M. penetrans* cells or that the His_6-tag fused to MYPE8180 in this study disturbed the recombinase activity. However, it is also possible that MYPE8180 has no activity in *mpl* promoter inversions, but is involved in another site-specific recombination system of the *M. penetrans* genome. It is known that several mycoplasma species such as *Mycoplasma genitalium* and *M. pneumoniae* lack *xer*-like site-specific recombinase genes, possibly because of reductive evolution of their minimum genomes (approx. 580 and 816 kb in size) (Himmelreich *et al.*, 1997). This fact suggests that these mycoplasma species, as a result of reductive evolution, do not require Xer-like recombinase for resolving their dimer chromosomes during cell division (Glew *et al.*, 2002; Recchia & Sherratt, 1999). However, the genome of *M. penetrans* (1.36 Mb) is approximately twice as large as that of these species. Furthermore, MYPE8180 shows strong homology to the XerC/D group of the Tsr family proteins. Thus, there is a possibility that MYPE8180 is involved in a Xer-like site-specific recombination system for chromosome segregation in *M. penetrans*. There is a further possibility that MYPE8180 is involved in another phase or antigenic variation system. To examine these possibilities, identification of the target sequence of MYPE8180 would be an interesting future study.

Identification of MYPE2900 recombinase in this study may lead to further research on disruption of the MYPE2900 gene and development of phase-variation-deficient (phase-locked) strains of *M. penetrans*. Such phase-locked strains were recently developed in *M. agalactiae*, a ruminant pathogen that has a multiple site-specific recombination system responsible for its surface lipoprotein variation (Chopra-Dewasthaly *et al.*, 2008). Phase-locked strains are useful for investigating host–pathogen interactions and for determining the functions of each lipoprotein variant. Although a gene disruption method needs to be developed in *M. penetrans*, an MYPE2900-deficient mutant might be developed in future studies. These attempts may provide an insight into the extent to which surface antigenic variations of mycoplasmas are involved in host immune evasion and help to establish their parasitic lifestyle. An understanding of these pathogenic mechanisms of mycoplasmas may be helpful for designing strategies to eradicate chronic mycoplasma infection.

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An improved abnormal toxicity test by using reference vaccine-specific body weight curves and histopathological data for monitoring vaccine quality and safety in Japan

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Abstract

Vaccines differ from other pharmaceutical products. The quality and safety of batches are regulated to high standards by national regulatory authorities. Various quality control and safety tests have been developed, including the abnormal toxicity test (ATT), which is described in the World Health Organization (WHO) guidelines and in each country's pharmacopoeia. However, the criteria for abnormal results are not well defined in these guidelines. In addition, the animal grade to be used in ATT, classified on the basis of microbial colonization, was not designated in either guideline.

In this study, we report a new and improved method of performing ATT, including statistical, histopathological analysis and hematological findings. It is based on the observation that there are body weight changes characteristic to each vaccine, and such standardized changes can be used as references for evaluating test vaccines. In addition, histopathological data are useful for determining vaccine quality and safety. Combined with histopathological examination, the improved ATT will be of great use for evaluating the consistency, quality and safety of different batches of vaccine. The results of these analyses were similar using either 'clean' or specific pathogen-free guinea pigs.

Keywords: Abnormal toxicity test; General safety test; Vaccine; Quality control

1. Introduction and history

Vaccination is one of the most effective and beneficial strategies for preventing and controlling human infectious diseases around the world. Various vaccines have been developed and can successfully prevent infectious diseases such as diphtheria, Haemophilus influenza b (Hib), pertussis, tetanus, polio, and influenza [1]. Vaccines are considered to differ from other pharmaceutical products because they are primarily intended for use in healthy people, including children. Vaccine production processes are also highly variable, depending on the target infectious disease. Current vaccines consist of inactivated, killed or live attenuated, antigenic components purified directly from organisms or produced by recombinant DNA technology. In addition, adjuvant is added to some vaccines to induce increased immunogenicity. On account of the complex production processes, vaccines are regulated by the national regulatory authorities of individual countries, on a lot-to-lot or batch-to-batch basis, and require high levels of quality and safety control.

To control and maintain vaccine quality, various quality control and safety tests have been developed, and are used to

Abbreviations: ATT, abnormal toxicity test; MRBP, minimum requirements for biological products; CFR, Code of Federal Regulations; SPF, specific pathogen-free.

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test the starting materials, materials under processing and the final products [2]. Among them, the abnormal toxicity test (ATT) (also known as the general safety test, innocuity test, or test for freedom from abnormal toxicity) has been developed to detect non-specific toxicity and/or contamination exogenous substances, and its inclusion in the national vaccine control regime is recommended by the WHO [3]. Many countries, including Japan [4] and the United States (21 CFR 610.11) [5], (but not EU countries) require ATT as a batch release testing to be performed on mice or guinea pigs for vaccines, sera and immunoglobulins [6]. In the EU, ATT is still included in some pharmacopeia European monographs and is required for the licensing and product validation process.

According to the WHO guidelines [3], the method of ATT is simple: 5 ml of vaccine is injected intra-peritoneally into guinea pigs and the animals are observed for 7–12 days. If there are no abnormal health signs, the product passes the test. The most frequently used signs to determine the safety of the vaccine are death rate and abnormal body weight changes.

In Japan, ATT was introduced in 1948 after immunization with a certain lot of diphtheria toxoid caused severe adverse events claiming 84 deaths in Kyoto and Shimane prefectures [7]. Since then, ATT has been conducted to test the safety of vaccine and blood products, both by the manufacturers and by the National Regulatory Authority, National Institute of Infectious Diseases (NIID).

ATT was described in the "minimum requirements for biological products (MRBP)" in article 42(1) of the Pharmaceutical Affairs Law in Japan, which defines the manufacturing methods, quality control and test methods applicable to pharmaceuticals [4]. According to the MRBP, the guinea pigs are required to survive without any abnormal signs during the observation and the body weight should return to its starting level within a specified time [8]. However, the criteria for abnormal signs are not detailed in either guideline. In addition, the required grade (defined by microbial colonization) of guinea pigs to be used in ATT is not designated in these guidelines. It has been suggested that animal grade is important in order to minimize the variation between test results and the effects of infectious agents on experimental results. When ATT was introduced in Japan in 1948, hemolytic streptococcal infection in guinea pigs was one of the most serious problems affecting body weight changes and test results. In 1971, a 'clean' grade of animals derived from specific pathogen-free (SPF) guinea pigs were introduced in ATT, which cleared problems caused by the infections. Nevertheless, it was thought that the clean grade guinea pigs were generated from SPF animals and were maintained under the SPF condition; they were fed with non-sterilized food and not monitored regularly for pathogens. Ideally, SPF and inbred animals should be used for ATT [9] and better methods could reduce the number of animals needed for research [10]. In Japan, inbred guinea pigs sufficiently well-characterized with respect to vaccine quality control testing are not available. In addition, it has been suggested that conventional guinea pigs are not suitable for evaluating liver toxicity, due to the natural occurrence of focal liver necrosis in clinically normal guinea pigs [11,12]. Thus, a change from "clean" to SPF animals for ATT is urgently required in Japan.

In this study, we report an improved method of ATT, including statistical and histopathological analyses. The trend in body weight changes following the inoculation of each vaccine, together with statistical and pathological analyses, were used to evaluate consistency among different vaccine batches. We also report that body weight changes and histopathological trends were not affected by changing the animal grade.

2. Materials and methods

2.1. Animals

According to the minimum requirements for biological products (MRBP) in Japan [4], we purchased female Hartley guinea pigs (280–300 g body weight, 'clean' grade and SPF grade) from SLC (Shizuoka, Japan). Both clean and SPF guinea pigs were derived from same Nakaizu colony. They were housed in solid-bottomed metal cages and fed on standard guinea pig chow. The temperature was maintained between 24–26 °C. Five guinea pigs were caged together during the tests. All of our experiments were performed as national control tests for vaccines and in accordance with the guidelines for animal experiments of the National Institute of Infectious Diseases, Japan.

2.2. Abnormal toxicity test (ATT)

Prior to ATT, the body weights of the guinea pigs (clean grade and SPF grade) were monitored for 7 days. Only animals with no abnormal signs were used for the test. Each injection group consisted of two animals selected randomly from the healthy animals. Vaccines (Table 1) at a dose of 5 ml were injected intra-peritoneally into each guinea pig. Vaccines used in this study were test specimens for vaccine testing and some vaccines were provided from manufactures for detailed examination. Body weight was monitored for 7 days after the injection. At day 7, the guinea pigs were anesthetized with pentobarbital (50 mg/kg) and processed for histopathological analysis. For hematological analysis, 2 ml peripheral blood was collected from the heart and immediately submitted to automatic measurements of peripheral blood leukocytes (PBL), mean corpuscular hemoglobin (HGB), red blood cells (RBC), hematocrit (HCT), mean corpuscular volume (MCV) and platelets (PLT) by automatic hematocytometer, the Celltac MEK-5254 (Nihon Kohden, Tokyo, Japan).

Animals were considered to have passed the test if there was no abnormal histopathological changes and no significant difference ($P < 0.01$) in the body weight curves between the test vaccine and reference population.

2.3. Statistical analysis

Significant differences were calculated using the z-test for the body weight changes and Student's t-test for other

Table 1
Body weight change after inoculation of various vaccines

Biological name	N	Body weight change after inoculation							
		Day 1		Day 2		Day 3		Day 4	
		AVE	VAR	AVE	VAR	AVE	VAR	AVE	VAR
Control (non-treatment, NT)	138	2.065	40.777	11.261	43.056	19.355	61.807	52.297	175.145
Control (saline, SA)	68	−2.382	23.046	8.544	35.714	16.176	36.386	48.456	147.326
Influenza HA vaccine (Flu-HA)	460	−5.098	59.160	8.148	63.372	16.467	65.914	52.507	173.819
Adsorbed diphtheria-purified pertussis–tetanus combined vaccine (DPT)	274	−4.704	40.495	6.215	44.199	14.712	61.898	47.588	179.100
Japanese encephalitis vaccine (JEV)	248	−9.734	45.370	6.520	46.639	15.210	47.470	47.895	148.119
Adsorbed hepatitis B vaccine (CHO) (HBV)	81	−6.074	27.119	6.568	35.248	14.074	62.519	50.235	168.307
Adsorbed tetanus toxoid (ATT)	79	−3.633	51.671	7.544	68.636	15.759	88.236	49.595	200.039
Adsorbed diphtheria–tetanus Combined Toxoid (ADCT)	55	−6.164	54.325	4.709	53.877	14.218	69.433	47.545	192.808
Pneumococcal vaccine polyvalent (PVP)	48	−38.813	65.092	−44.375	90.282	−27.021	97.340	21.667	101.163
Cholera vaccine (CV)	44	−14.568	79.879	−3.045	106.789	6.227	54.087	38.000	179.349
Freeze-dried inactivated tissue culture rabies vaccine (RV)	29	−11.241	40.047	4.276	68.778	12.448	68.399	43.345	105.448
Freeze-dried inactivated tissue culture hepatitis A vaccine (HAV)	28	−0.964	58.554	9.107	39.284	14.357	38.608	47.321	142.078
Yellow fever vaccine (YFV)	12	−1.000	24.545	10.917	24.992	18.500	47.364	55.417	142.629
Well's disease and Akiyami combined vaccine (WDACV)	12	−15.083	85.174	−7.750	171.477	2.917	101.902	37.583	341.174
Adsorbed habu-venom toxoid (HT)	10	−7.000	33.333	3.800	22.400	10.700	23.789	40.500	134.722
Adsorbed diphtheria toxoid for adult use (ADT)	8	−12.750	36.500	−0.375	42.554	7.250	42.214	47.000	143.143
Freeze-dried mamushi antivenom, equine (MA)	6	−1.500	16.300	6.667	47.867	14.500	30.300	51.833	224.967
Freeze-dried habu antivenom, equine (HA)	6	2.500	14.300	14.167	116.167	25.833	198.167	55.667	435.067
Freeze-dried botulism antitoxin, equine (BA)	4	1.500	13.667	12.000	22.000	18.750	18.917	56.250	47.583
Freeze-dried gas gangrene Antitoxin, equine (GGA)	2	−8.000	128.000	14.500	60.500	23.000	0.000	53.500	112.500
Gas gangrene antitoxin, equine (GGAe)	2	8.000	2.000	16.000	18.000	22.500	84.500	38.500	40.500

The average (AVE) and variance (VAR) of body weight change 1, 2, 3, and 7 days after various vaccines treatment were shown.

statistical analyses, according to the MRBP in Japan [12]. To evaluate difference between clean grade and SPF grade guinea pigs, Spearman's rank correlation coefficient (r) was calculated. All statistical analyses were performed using GraphPad Prism (version 4, GraphPad Software, San Diego, CA), Excel 2008 (Microsoft Japan, Tokyo, Japan) and JMP Statistical Software (version 5, SAS Institute Inc., Cary, NC).

2.4. Histology

At day 7 after vaccination, animals were anesthetized with pentobarbital (50 mg/kg). Afterwards, pancreas, spleen and liver specimens were excised and weighed. Tissues were fixed in Bouin's solution (Sigma, St. Louis, MO) and 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C for 24 h. After fixation, tissues were dehydrated through a series of graded ethanols and xylene and embedded in paraffin. Paraffin-embedded specimens were cut into 4 μm sections and stained with hematoxylin and eosin. The surface area of focal necrosis and aluminum deposition were measured using a slide gauge.

3. Results

3.1. Selection of healthy animals in the preliminary test

Prior to performing ATT, guinea pigs (280–300 g body weight) were monitored for 7 days under normal conditions (Fig. 1A). Fig. 1B shows the data of body weight change monitored for 7 days before the start of ATT, which was obtained from previous tests done in NIID using 8000 guinea pigs. As shown in Fig. 1C, the increase in body weight followed a linear regression ($Y = 14.899X - 13.054$, $R^2 = 0.9655$) and the daily increase in body weight was normally-distributed at days 2 and 3 after the animals arrived at the laboratory. Body weights were increased by 10.87 ± 6.58 g at day 2 and by a further 7.963 ± 5.51 g at day 3. Body weight changes at day 1 were highly variable due to transportation stress and dietary conditions prior to delivery. Thus, we have not taken body weight changes at day 1 into consideration (data not shown). Based on these preliminary test data above, we judged and selected animals with coefficients of regression of 5 and coefficients of correlation to the parent population of 0.866 as being suitable for further experimentation. After selection, the guinea pigs were classified according to their final body weights and randomized to different vaccine-treated groups. Vaccines was injected intra-peritoneally and body weights were monitored for the next 7 days.

3.2. Body weight changes after vaccine injection

Table 1 shows types of vaccines used in this study. We used two control groups, non-treated (NT) and saline-treated (SA) one. Fig. 2A-1 shows body weight changes after the vaccine injection over the 7 day period. In the NT group, body weight increased by 2.065 g at day 1, then by 11.261 g (day 2), 19.355 g (day 3), and 52.297 g (day 7) (Fig. 2A). In the SA group, body weight initially decreased by 2.382 g at day 1, but then increased by 8.544 g (day 2), 16.176 g (day 3), and 48.456 g (day 7) (Fig. 2B). As in the SA group, body weights

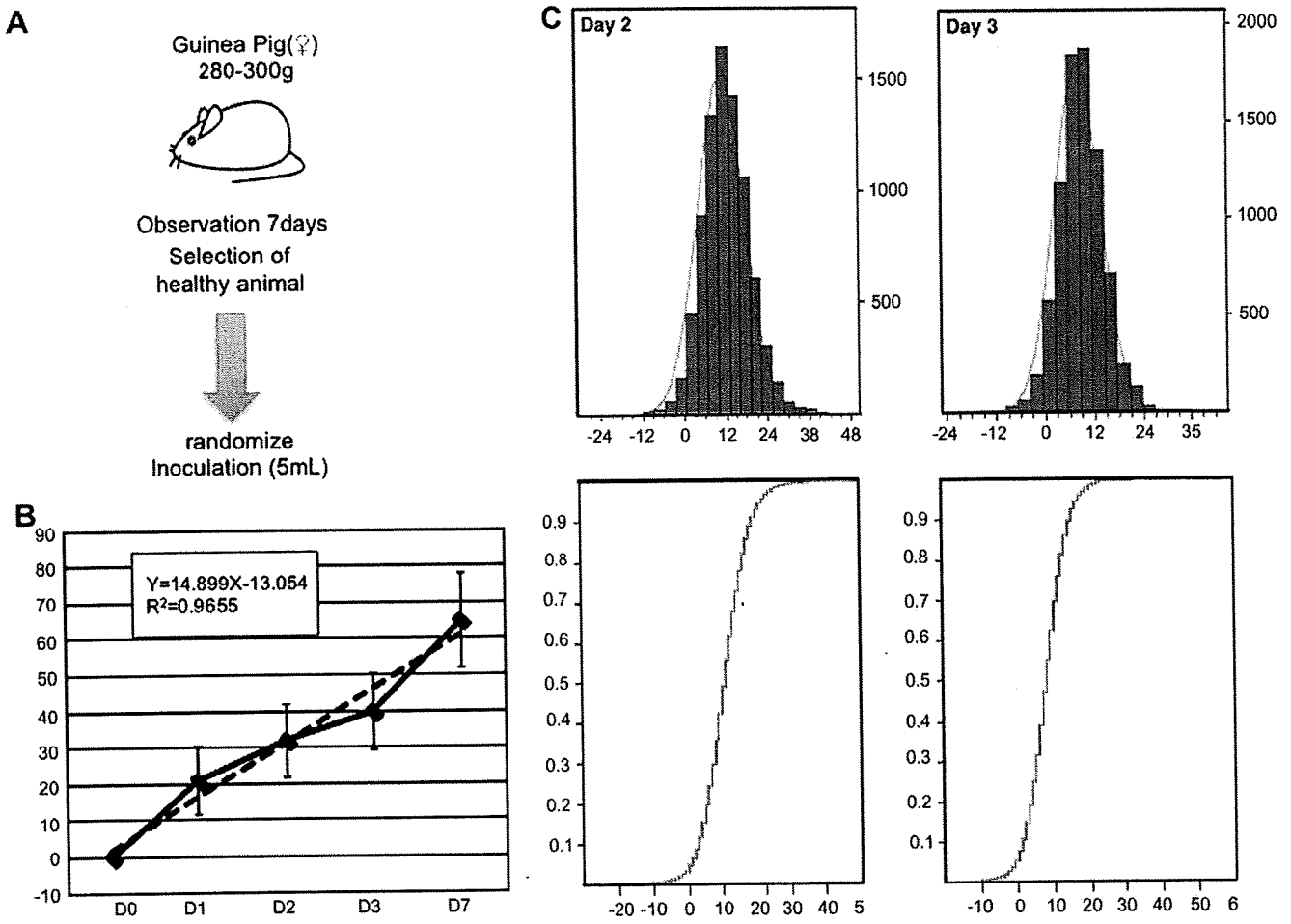

Fig. 1. Preliminary phase of abnormal toxicty test. (A) Summary of preliminary test. (B) Body weight changes after the arrival of guinea pigs at our animal facility. Collinear approximation is $Y = 14.899X - 13.054$ and coefficient of determination $R^2 = 0.9655$. (C) Upper panel: normal distribution of daily body weight change at day 2 and 3. Lower panel: cumulative distribution of daily body weight changes at day 2 and 3.

initially declined in several vaccine-treated guinea pigs: −5.098 g (influenza vaccine;; Flu-HA), −4.704 g (adsorbed diphtheria-purified pertussis–tetanus combined vaccine, DPT), −9.734 g (Japanese encephalitis vaccine, JEV), −6.074 g (recombinant adsorbed hepatitis B vaccine, HBV), −3.633 g (adsorbed tetanus toxoid, TT), −6.164 g (adsorbed diphtheria–tetanus combined vaccine, ADCT), and −38.813 g (pneumococcal vaccine polyvalent, PCV) (Fig. 2C-J). At day 2, body weights increased to reach and exceed the initial body weights in the Flu-HA-, DTP-, JEV-, HBV-, TT- and ADCT-treated guinea pigs. Body weights continued to decline at day 2 only in the PVP-treated guinea pigs (−44.375 g), and although body weights had increased by day 3, they had still not reached the initial body weight. At day 7, body weights had increased and recovered to the initial body weights in the PVP-treated guinea pigs. These trends in body weight changes followed a specific pattern with an initial reduction in body weight followed by an increase to the original body weight, with the increase being almost linear. Body weight changes at day 1 for various vaccines are summarized in Fig. 2J. Using the body weight curve data for each vaccine of at least 50 batches of vaccine, we constructed standard body weight change values for each vaccine. The population of animals with these values are known as the "reference population" (RP) (Table 1). Deviations from RP values were calculated by z-test, and if the values are within the reference range ($P < 0.01$) and no pathological signs were evident, we judged that the test specimen passed ATT. If the body weights fell below the reference values, ATT was repeated twice more before final judgment.

3.3. Pathological changes in vaccine-treated guinea pigs

Pathological changes, such as hemorrhagic ascites (Fig. 3A) and inflammation of the pancreas (Fig. 3B, C) were seen with guinea pigs receiving DPT, aluminum adsorbed vaccine and toxoid. Such changes were associated with a significant decrease of the body weight in comparison with RP ($P < 0.01$). However, the pathological effects varied among different batches of vaccines. The statistical analysis of ATT results improved the identification of pathological changes in the liver, spleen and pancreas. We measured the number of leukocytes (Fig. 3F), pancreas (Fig. 3G) and spleen weights (Fig. 3H), the area of focal necrosis in the liver (Fig. 3I) and the area of aluminum deposition in the pancreas (Fig. 3J), using more than 10 batches of each vaccine. These

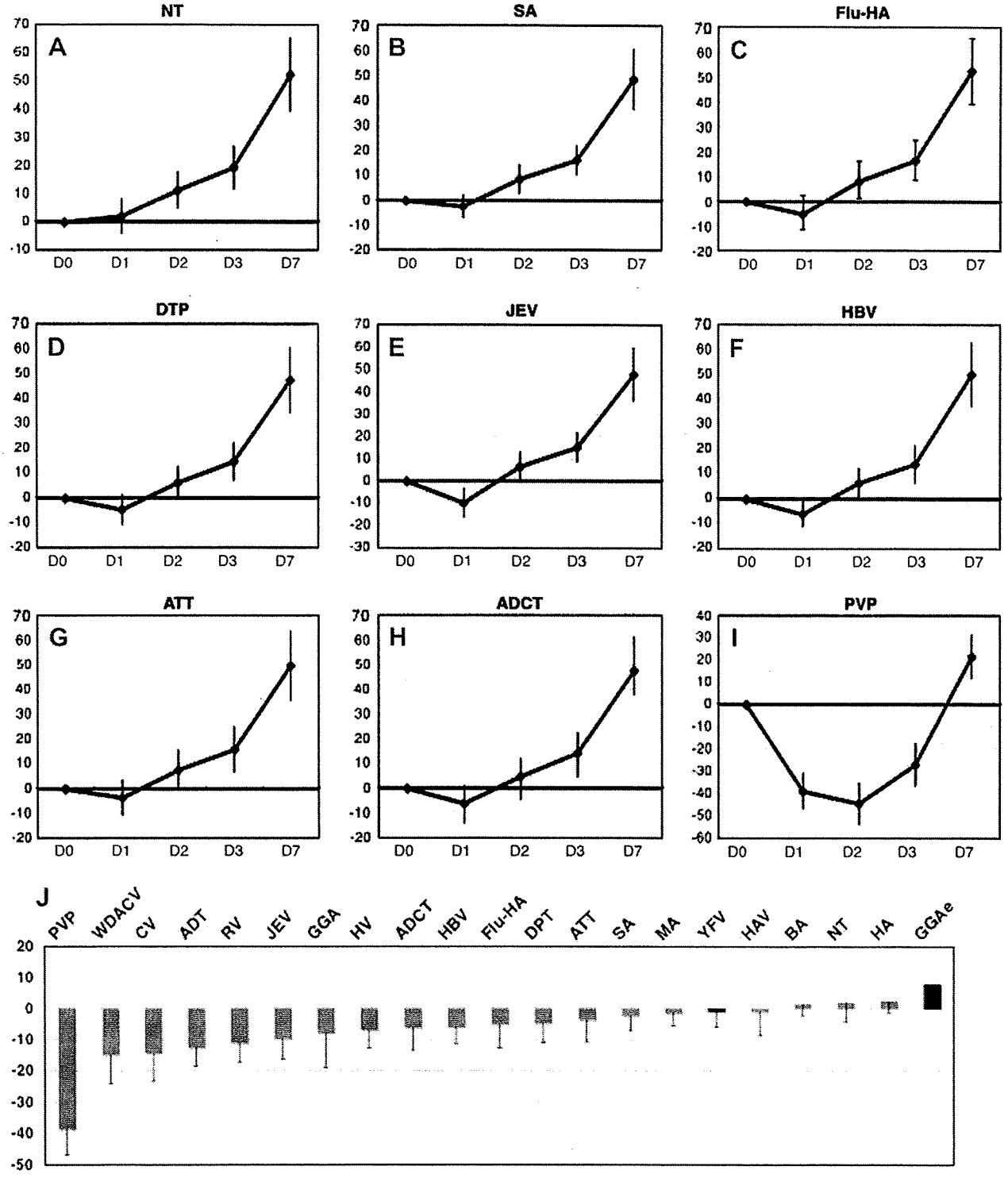

Fig. 2. Effects of several vaccine treatments on guinea pig body weight. Body weight changes after vaccination. (A) non-treated (NT) and (B) saline (SA)-treated animals as a control. (C) influenza HA vaccine (Flu-HA), (D) adsorbed diphtheria-purified pertussis-tetanus combined vaccine (DTP), (E) Japanese encephalitis vaccine (JEV), (F) hepatitis B vaccine (HBV), (G) adsorbed tetanus toxoid (TT), (H) adsorbed diphtheria-tetanus combined toxoid (ADCT), (I) pneumococcal vaccine polyvalent (PVP). (J) Body weight changes at day 1. WDACV, Weil's disease and Akiyami combined vaccine; CV, cholera vaccine; RV, rabies vaccine; GGA, freeze-dried gas gangrene antitoxin, equine; HV, adsorbed habu-venom toxoid; MA, freeze-dried mamushi antivenom, equine; HAV, freeze-dried inactivated tissue culture hepatitis A vaccine; BA, freeze-dried botulism antitoxin, equine; HA, freeze-dried habu antivenom, equine; GGAE, gas gangrene antitoxin, equine.

pathological changes were characteristic to each vaccine, and occurred not only in animals receiving the re-tested and rejected vaccines, but also in the passed vaccines, especially in the DPT, aluminum adsorbed vaccine and toxoid. The degree of pathological change varied, though no significant differences were found between controls and the test vaccines ($P < 0.05$). These data indicate that the pathological changes were due to the reactogenicity of the vaccines, rather than their

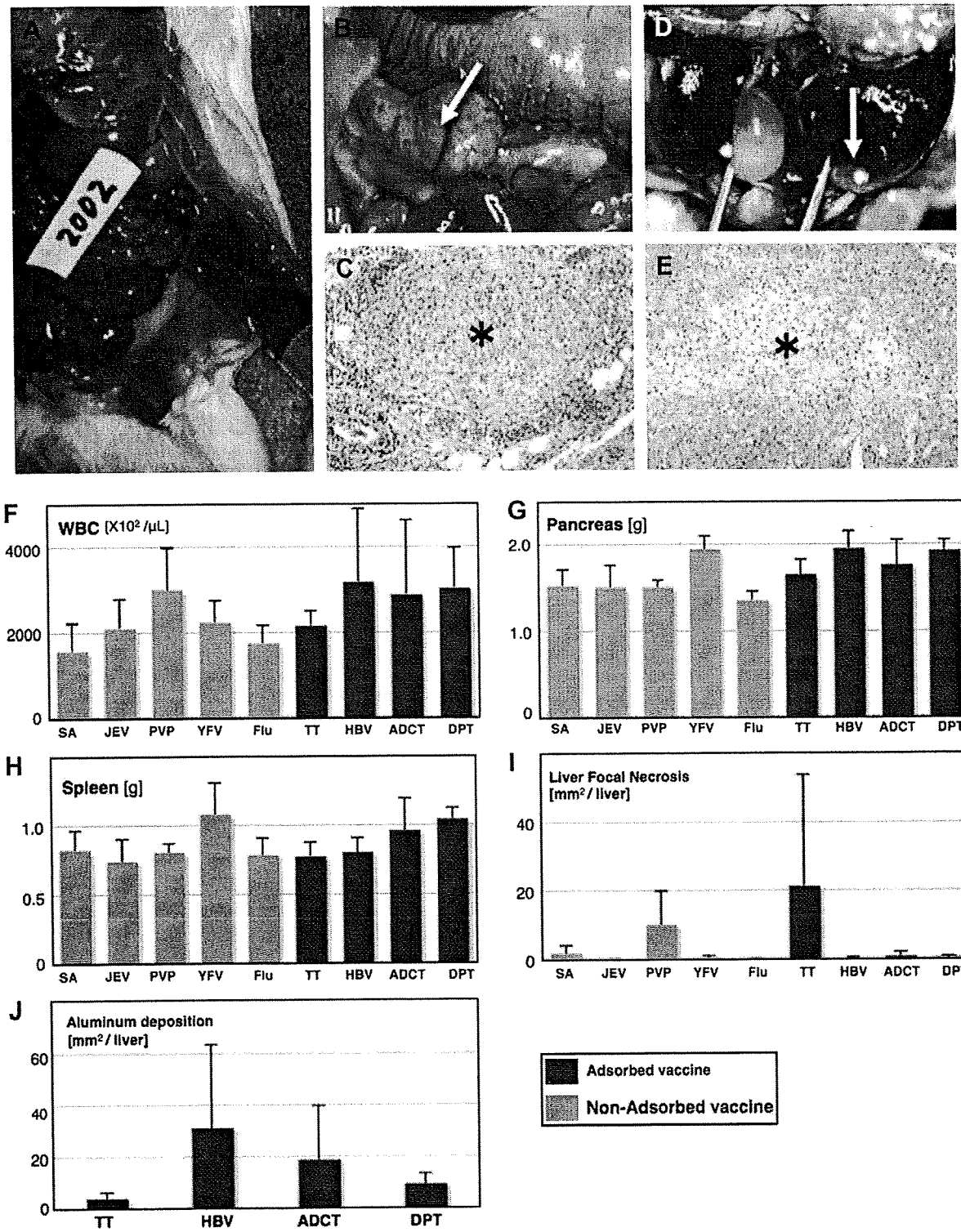

Fig. 3. Histopathological analysis in the guinea pig after vaccination. (A) Specific toxic and pathological changes, such as hemorrhagic ascites in the abdomen of the DPT-treated guinea pigs at day 7. (B) Inflammation in the DPT-treated pancreas at day 7. (C) Histological analysis of inflammation in the DPT treated pancreas by hematoxylin and eosin staining (H E). (D) Focal necrosis in the DPT-treated liver at day 7. (E) Histological analysis of focal necrosis in the DPT-treated liver. H E staining. (F) The number of leukocytes in the peripheral blood after vaccination at day 7. (G) Pancreas weight after vaccination at day 7. (H) Spleen weight after vaccination at day 7. (I) The area of focal necrosis in the vaccine-treated liver (mm^2). (J) Total area of aluminum deposition in the pancreas (mm^2). NT, non-treated; SA, saline; Flu-HA, influenza HA vaccine; DTP, adsorbed diphtheria-purified pertussis-tetanus combined vaccine; JEV, Japanese encephalitis vaccine; HBV, hepatitis B vaccine; TT, adsorbed tetanus toxoid; ADCT, adsorbed diphtheria-tetanus combined toxoid; PVP, pneumococcal vaccine polyvalent.

toxicity. These histopathological reference data will help us to define the criteria for abnormal toxicity levels in histopathological analyses.

3.4. Effects of animal grade change on ATT

To improve the quality of histopathological analyses, 'clean' animals need to be substituted by SPF animals. Our body weight RP was based on 'clean' animals, and we needed to determine the effect of changing the animal grade on the results of ATT. To validate the reliability and reproducibility of our reference population based on 'clean' animals, we focused on the vaccines (Flu-HA, DTP, YFV, JEV, HBV, TT, ADCT, PVP) for which a RP of body weights and histopathological features had already been established and confirmed in our current ATT.

Compared with the RP based on 'clean' guinea pigs, no significant differences in body weight changes were observed in NT, SA, Flu-HA, DTP, JEV, HBV, TT, ADCT and PVP-treated SPF guinea pigs ($P < 0.01$). Strong correlations between body weight changes in the 'clean' and SPF-based RPs were demonstrated (Fig. 4). Using the SPF-based RP, the conclusions from ATT based on the 'clean' RP were not changed.

3.5. Effects of animal grade change on pathological changes

To validate our modified ATT, we compared the data obtained with clean animals with those obtained with SPF animals. No significant differences in body weight changes were observed in NT, SA, Flu-HA, DPT, JEV, HBV, TT, ADCT and PVP-treated SPF guinea pig ($P < 0.01$) (Fig. 4). Pathological changes were similar for the both group injected with JEV, PVP, YFV, Flu-HA, TT, HBV, ADCT and DPT. The WBC (Fig. 5A), the pancreas (Fig. 5B) and spleen weights (Fig. 5C), and the area of focal necrosis in the liver (Fig. 5D) and area of aluminum deposition (Fig. 5E) were similar in both sets of animals ($P < 0.05$).

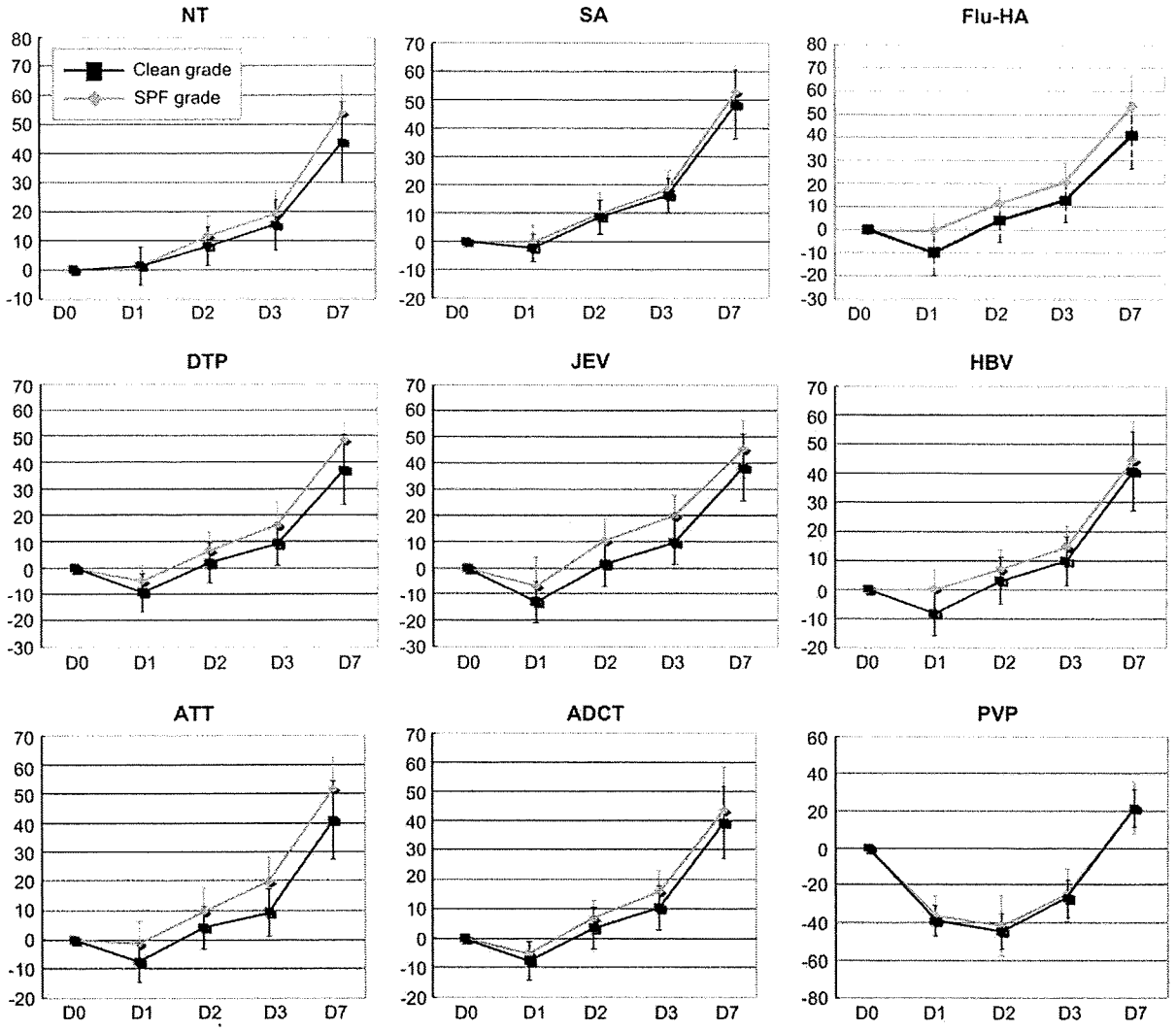

Fig. 4. Effects of animal grade changes from 'clean' to specific pathogen-free (SPF). The body weight changes after vaccination in the SPF reference group were similar to those in the 'clean' reference group. A strong correlation in body weight change was observed between the 'clean' and SPF-based reference populations. NT, non-treated; SA, saline; Flu-HA, influenza HA vaccine; DTP, adsorbed diphtheria-purified pertussis-tetanus combined vaccine; JEV, Japanese encephalitis vaccine; HBV, hepatitis B vaccine; TT, adsorbed tetanus toxoid; ADCT, adsorbed diphtheria-tetanus combined toxoid; PVP, pneumococcal vaccine polyvalent.

3.6. Proposal of improved ATT (Fig. 6)

The diagram of the improved protocol for ATT is shown in Fig. 6. It has two steps: first, arriving animals weighing 280–300 g were monitored for 7 days and only healthy animals are used for ATT. The animals are then randomly allocated to control and vaccine-treated groups, each consisting of two animals. After vaccine injection, the body weight is monitored for 7 days. If there is no significant deviation ($P < 0.01$) from RP values in Table.1, vaccines are considered to have passed the test. If a significant difference is found, histopathological analyses should be performed and the test should be repeated

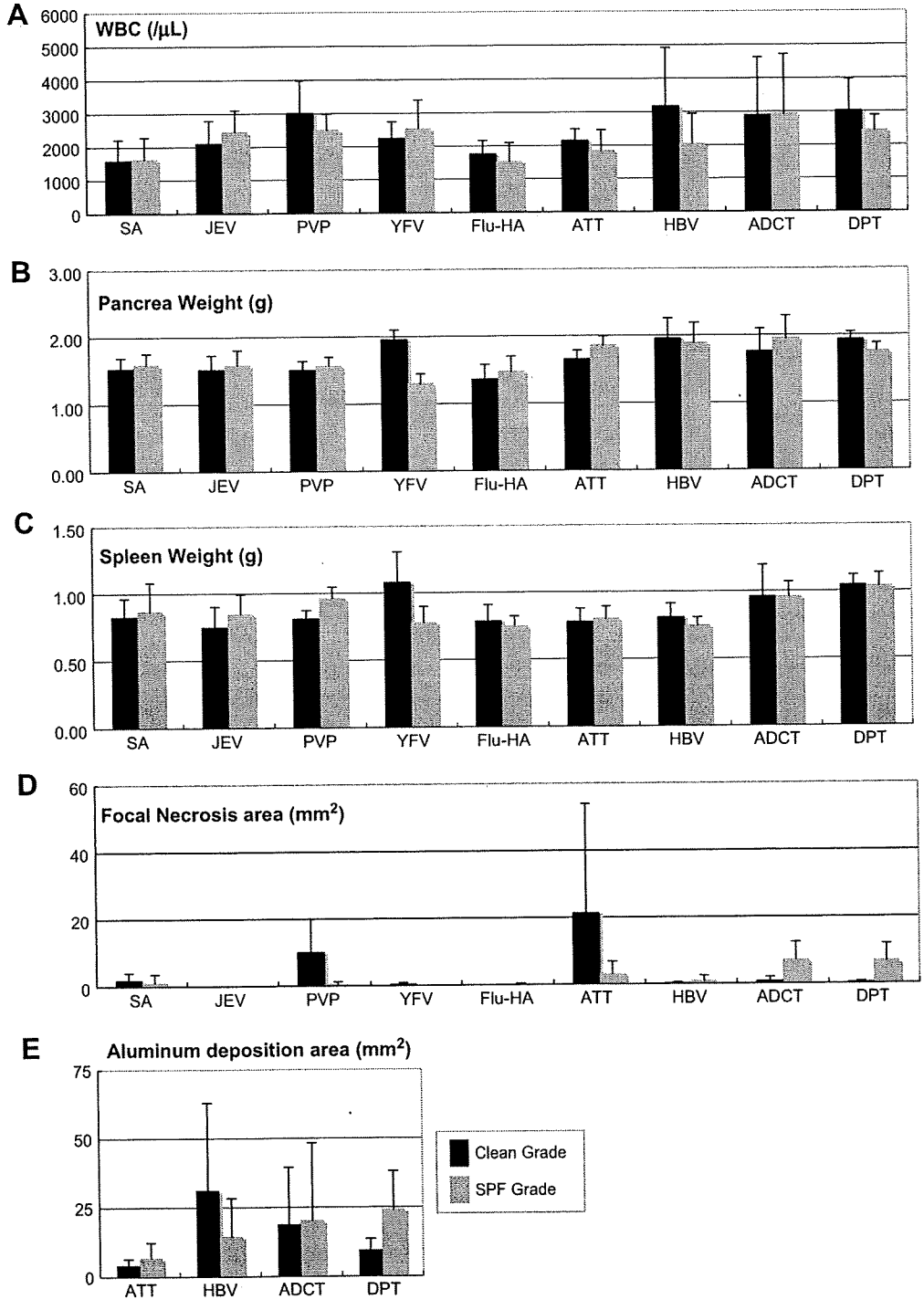

Fig. 5. Comparison of histopathological data between 'clean' and specific pathogen-free (SPF) reference groups. (A) The number of leukocytes, (B) pancreas weight, (C) spleen weight, (D) the area of focal necrosis in the liver, (E) total area of aluminum deposition in pancreas were similar in the 'clean' and SPF-based reference populations. NT, non-treated; SA, saline; Flu-HA, influenza HA vaccine; DTP, adsorbed diphtheria-purified pertussis-tetanus combined vaccine; JEV, Japanese encephalitis vaccine; HBV, hepatitis B vaccine; TT, adsorbed tetanus toxoid; ADCT, adsorbed diphtheria-tetanus combined toxoid; PVP, pneumococcal vaccine polyvalent; YFV, yellow fever vaccine.