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## ATTACHMENT OF ANTIGENIC VARIANTS OF LEPTOSPIRAS TO MOUSE FIBROBLASTS RESISTING INHIBITORY EFFECT OF ANTI-PARENT ANTISERUM

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Antigenic variants of *Leptospira interrogans* serovar *canicola* Moulton and *hebdomadis* Hebdomadis, which were isolated from the parental population by selection with monoclonal antibodies, were shown to attach to mouse skin fibroblast cells and extracellular matrix (ECM). The attachment of the variants occurred in the presence of anti-parent antiserum. Resistance of the variants to the effect of anti-parent antiserum in the attachment was in accord with their serological remoteness from the parent.

Key words: *Leptospira*, antigenic variant, attachment, mouse fibroblasts, extracellular matrix

### INTRODUCTION

Attachment of *Leptospira interrogans* to cells or tissues has been studied only recently. Virulent leptospires attached abundantly, while avirulent leptospires attached only poorly to culture cells<sup>14,15)</sup> and extracellular matrix (ECM).<sup>7)</sup> Antiserum against leptospires inhibited the attachment of homologous leptospires to the cells and ECM. Fab fragment of the IgG from the antiserum also inhibited the attachment.<sup>7,14)</sup>

Antigenic variants of leptospires have been selected by cultivating leptospires in the presence of homologous and heterologous antisera,<sup>1,2,9,10,11,18)</sup> factor sera<sup>3)</sup> and monoclonal antibody.<sup>12)</sup> Recently we found that a population of *L. interrogans* serovar *hebdomadis* contained a variety of antigenic variants in different ratios.<sup>6)</sup>

Vaccination has been considered an effective measure to prevent leptospiral infection; however, it is believed that the disease cannot be completely prevented by this method.<sup>13)</sup> The reasons for break of vaccination in leptospirosis are complex, one important one being the fact that antigenic variants are present in a leptospiral population. Such antigenic variants may multiply *in vivo*, especially when the infected animals possess some amount of antibodies against leptospires due to natural

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infection or vaccination. Thus it is interesting to study whether such variants attach to the cells and ECM in the presence of anti-parent antiserum.

The focus of the present communication is to examine the attachment of several antigenic variants derived from the parental population of *L. interrogans* serovars *canicola* and *hebdomadis* to mouse skin fibroblast cells and ECM, and to demonstrate the attachment of variants that resist the effect of anti-parent antiserum.

#### MATERIALS AND METHODS

TABLE 1 Strain used

Parents and variants	Virulence	50% agglutination with dilution of	
		Anti-Moulton	Anti-Hebdomadis
<i>canicola</i>	Hamster-lethal <sup>a</sup>		
Moulton	✓	6,400	
MoV(CT3)-1		100	
<i>hebdomadis</i>			
Hebdomadis	Non-virulent <sup>b</sup>		6,400 <sup>c</sup>
HV(H19)-1 <sup>d</sup>	✓		6,400 <sup>c</sup>
HV(H1)-1	✓		3,200 <sup>c</sup>
HV(H16)-1	✓		800 <sup>c</sup>
HV(H14)-1	✓		800
HV(H9)-1	✓		1,600

<sup>a</sup>: LD<sub>50</sub> was < 10<sup>8</sup>.

<sup>b</sup>: Non-lethal with intraperitoneal inoculation of 10<sup>8</sup>.

<sup>c</sup>: Data from ITO et al.<sup>6</sup>

<sup>d</sup>: HV(H19)-1, which was not agglutinated by monoclonal antibody H19, was serologically indistinguishable from the parent strain.

**Strains** Leptospiral strains used in the present study are listed in Table 1. As a parent for obtaining antigenic variants, virulent *canicola* Moulton and avirulent *hebdomadis* Hebdomadis, which were cloned, respectively, on agar medium<sup>4)</sup> were used. Moulton was passaged through hamsters several times a year and shown to be hamster-lethal.<sup>5,8)</sup> The antigenic variant designated MoV(CT3)-1 was isolated from Moulton by selection with anti-*canicola* monoclonal antibody CT3, according to the procedure described.<sup>12)</sup> The antigenic variant was hamster-lethal with intraperitoneal inoculation with 10<sup>8</sup> organisms/ml. Anti-Moulton antiserum with the homologous 50% microscope agglutination end point titer of 1: 6,400 agglutinated MoV(CT3)-1 only at 1: 100. Serovar *hebdomadis* strain Hebdomadis and the 5 antigenic variants isolated from Hebdomadis by selection with anti-Hebdomadis monoclonal antibodies H1, H19, H16, H14 and H9, respectively, which were designated HV(H1)-1, HV(H19)-1,

HV(H16)-1, HV(H14)-1 and HV(H9)-1, respectively,<sup>6)</sup> were also used. All variants isolated from *Hebdomadis* were non-virulent. Anti-*Hebdomadis* antiserum with the homologous titer of 1: 6,400 agglutinated 4 variants at the decreased titers of 1: 3,200 (HV(H1)-1), 1: 800 (HV(H16)-1), 1: 800 (HV(H14)-1) and 1: 1,600 (HV(H9)-1). Another variant, HV(H19)-1, which was isolated by selection with anti-parent monoclonal antibody H19 and not agglutinated by the monoclonal antibody, was agglutinated by anti-parent antiserum at the titer of 1: 6,400 similar to the parent.

**Cells and ECM** Secondary mouse skin fibroblasts were used for attachment tests. The culture of mouse skin fibroblasts was prepared as follows. Skin (5×5mm) of newborn BALB/c mice was removed and washed 3 times sequentially with Dulbecco's modified Eagle's medium (DMEM) with antibiotics. The skin was then cut into 1×1mm pieces with a sterile scalpel blade, and 4-5 pieces of skin were put into plastic plates. The plates were air dried for 10 min and 5ml of DMEM containing 10% fetal bovine serum were added. After incubation at 37°C in a humidified CO<sub>2</sub> atmosphere for 7-14 days, fibroblastic cells grew and covered entire plate. The cells were distributed into five glass plates containing coverslips after dissociation of cell monolayers with 0.25% trypsin in phosphate buffered saline (PBS, pH 7.2). ECM preparations were obtained from the cell cultures on coverslips by treatment with 0.5% Triton X-100 for 10 min at room temperature. Coverslips were then washed 3 times with PBS before use.

**Attachment test** Attachment tests of antigenic variants to culture cells and ECM were done as previously described.<sup>7,14)</sup> Briefly, a volume of 1 ml of leptospiras at 10<sup>8</sup> organisms/ml was added to the coverslips placed in 24-well multiplates. The plates were incubated for 2 hr at 30°C. After incubation, the coverslips were drained, gently rinsed 7-8 times in PBS to remove non-adhering leptospiras and placed on slides. The number of leptospiras that were attached to 20 randomly selected individual cells or spots of ECM was counted at a magnification of × 600 under dark-field microscopy.

**Immune serum** The hyperimmune rabbit sera against *canicola* Moulton and *hebdomadis* *Hebdomadis* were prepared as previously described.<sup>17)</sup>

**Inhibition of leptospiral attachment by immune serum** Two-fold-serial dilutions of anti-sera were prepared. 0.5ml of each dilution of anti-serum was mixed with an equal volume of suspension of each leptospira (1×10<sup>8</sup>/ml of final population) and incubated for 1 hr at room temperature. Then the mixture was added to cultured cells or ECM and incubated for 2 hr at 30°C. The number of attached leptospiras was counted as described above.

## RESULTS

Serovar *canicola* strain Moulton attached to mouse skin fibroblasts and ECM at 20.3 organisms per cell and 37.2 organisms per spot of ECM, respectively (Tab. 2).

TABLE 2 Attachment of antigenic variants of leptospiras to mouse skin fibroblasts and ECM<sup>a</sup>

Strains	Numbers of attached leptospiras (Mean $\pm$ S.E.)	
	Cells	ECM
<i>Canicola</i>		
Moulton	20.3 $\pm$ 11.4 <sup>b</sup>	37.2 $\pm$ 15.2 <sup>c</sup>
MoV(CT3)-1	19.7 $\pm$ 9.3	35.6 $\pm$ 8.9
<i>hebdomadis</i>		
Hebdomadis	9.3 $\pm$ 5.3	15.8 $\pm$ 5.9
HV(H19)-1	8.2 $\pm$ 4.2	14.1 $\pm$ 6.3
HV(H1)-1	10.4 $\pm$ 3.7	17.0 $\pm$ 5.1
HV(H16)-1	8.7 $\pm$ 4.8	14.4 $\pm$ 4.6
HV(H14)-1	9.5 $\pm$ 6.3	13.6 $\pm$ 5.7
HV(H9)-1	7.8 $\pm$ 2.9	15.4 $\pm$ 3.8

<sup>a</sup> Co-incubation for 2 hr at 30°C.<sup>b</sup> Attached leptospiras per cell. Twenty cells were examined.<sup>c</sup> Attached leptospiras per spot of ECM. Twenty spots of ECM were examined.

Number of antigenic variant, MoV(CT3)-1, that attached to the cells and ECM was almost similar to the number of the parent that attached to the cells and ECM. Serovar *hebdomadis* strain Hebdomadis attached to the cells and ECM at 9.3 per cell and 15.8 per spot of ECM, respectively. Five antigenic variants attached similarly in number, ranging from 8.2 to 10.4 per cell and from 13.6 to 17.0 per spot of ECM. Thus, antigenic variants attached similar in number as the parent did to the cells and ECM.

Attachment to the cells of *canicola* Moulton and the antigenic variant MoV(CT3)-1 in the presence of the anti-parent antiserum was compared (Fig. 1). The attachment of Moulton was inhibited in the presence of homologous antiserum (Fig. 1a). No attachment was observed at the dilution of 1: 200-800. But at the dilution of 1: 6,400, the attachment of Moulton was enhanced. At the dilution of 1: 1,600, the number of attached organisms was uncountable because many clumps of agglutinated leptospiras covered the cells. In the presence of normal rabbit serum, about 17 organisms per cell of Moulton attached to the cells (Fig. 1a). The attachment of MoV(CT3)-1 in the presence of anti-parent antiserum was not inhibited (Fig. 1b). Enhancement of the attachment of MoV(CT3)-1 by immune serum was observed at the dilution of 1: 200-800. In the presence of normal rabbit serum, the number of MoV(CT3)-1 that attached to the cells was about 18, similar to the number of the parent that attached to the cells.

In the presence of anti-MoV(CT3)-1 antiserum at the titer of 1: 6,400, the attachment of the variant was inhibited (data not shown).

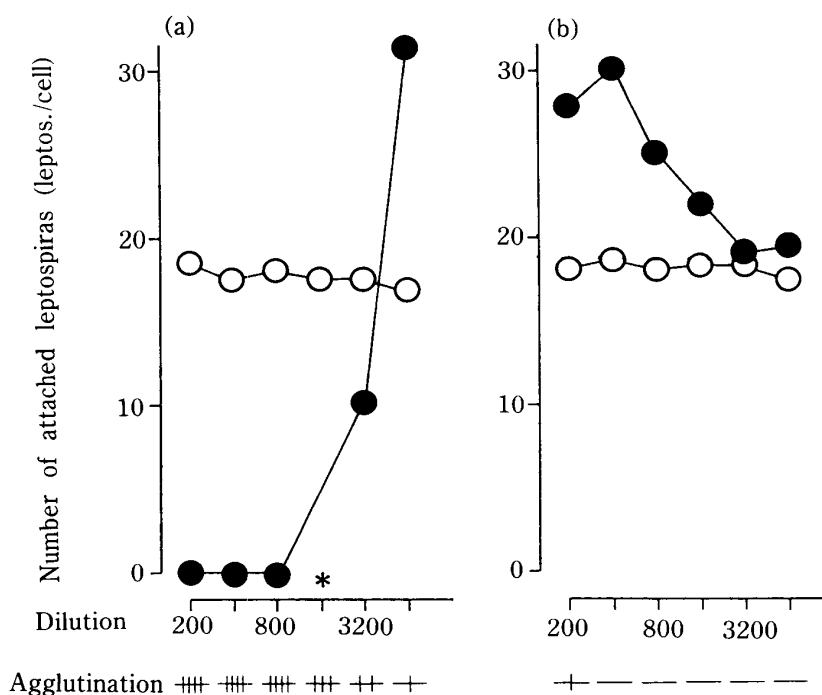


FIGURE 1 Attachment of antigenic variant and parent to mouse skin fibroblast cells in the presence of antiserum against parent. (a) Moulton (parent) (b) MoV(CT3)-1 Symbols: (○) normal rabbit serum (●) anti-Moulton antiserum. Degree of microscopic agglutination; ++++(100%) +++(75%) ++(50%) +(25%) -(0%) \* indicates attachment of leptospires was undetectable because of many clumps agglutinated leptospires.

Attachment to the cells of *hebdomadis* Hebdomadis and the five antigenic variants in the presence of the anti-Hebdomadis antiserum is shown in Figure 2. The attachment of Hebdomadis was completely inhibited in the presence of homologous antiserum (Fig. 2a). The attachment of HV(H19)-1, which was serologically indifferent from the parent, was inhibited by anti-Hebdomadis antiserum, similarly to the inhibition of attachment of the parent (Fig. 2b). The remaining 4 variants, which were serologically different from the parent, attached to the cells in various degrees in the presence of anti-Hebdomadis antiserum (Figs. 2c-f). Enhancement of attachment was observed in each variant at the dilution of antiserum showing weak agglutination. In the presence of normal rabbit serum, about 6-9 organisms per cell of each variant attached to the cells.

Similar results were obtained in the attachment of the variants to ECM in the presence of anti-parent antiserum (Figs. 3 & 4). The attachment of Moulton was inhibited by anti-Moulton antiserum (Fig. 3a), but the attachment of the variant,

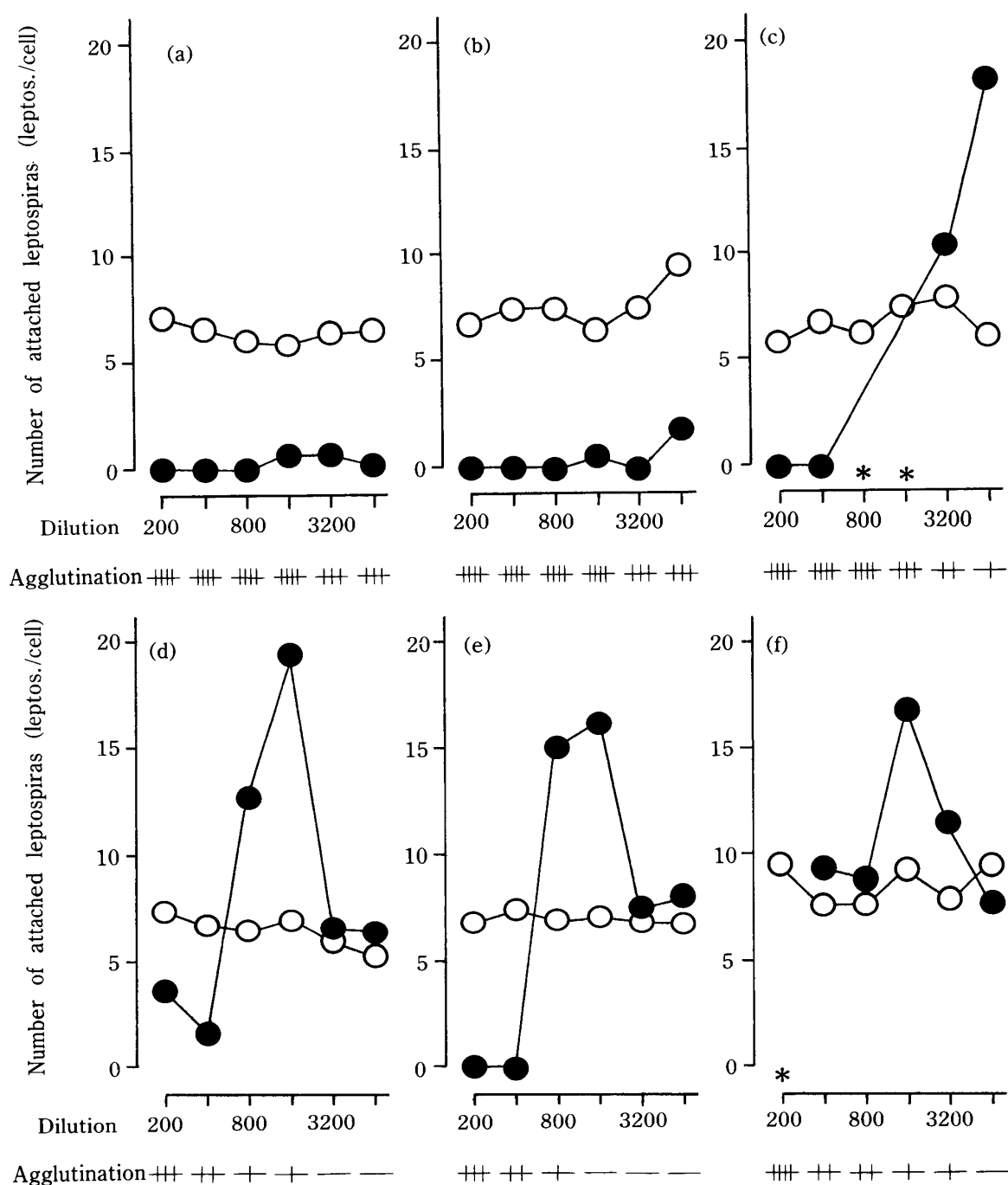


FIGURE 2 Attachment of antigenic variant and parent to mouse skin fibroblast cells in the presence of antiserum against parent. (a) Hebdomadis (parent) (b) HV(H19)-1 (c) HV(H1)-1 (d) HV(H16)-1 (e) HV(H14)-1 (f) HV(H9)-1 Symbols: (○) normal rabbit serum (●) anti-Hebdomadis antiserum. Degree of microscopic agglutination; +++++ (100%) +++ (75%) ++ (50%) + (25%) — (0%) \* indicates attachment of leptospiras was undetectable because of many clumps of agglutinated leptospiras.

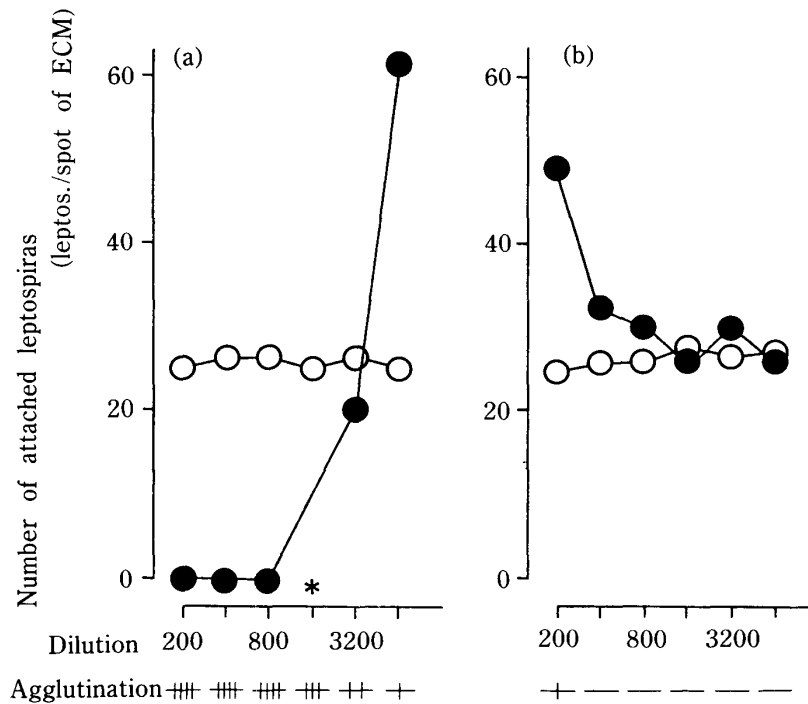


FIGURE 3 Attachment of antigenic variant and parent to ECM of mouse skin fibroblast cells in the presence of antiserum against parent. (a) Moulton (parent) (b) MoV(CT3)-1 Symbols: (○) normal rabbit serum (●) anti-Moulton antiserum. Degree of microscopic agglutination; +++ (100%) ++ (75%) + (50%) + (25%) - (0%) \* indicates attachment of leptospires was undetectable because of many clumps of agglutinated leptospires.

MoV(CT3)-1, was not inhibited by the same antiserum (Fig. 3b). The attachment of Hebdomadis and the variant HV(H19)-1 was inhibited by anti-Hebdomadis antiserum (Figs. 4a & 4b). The inhibitory effects of anti-parent antiserum were minimized in the other 4 antigenic variants in accord with their weak agglutinability or serological remoteness from the parent (Figs. 4c-f). Enhancement of attachment was also observed in each variant at the dilution of antiserum showing weak agglutination.



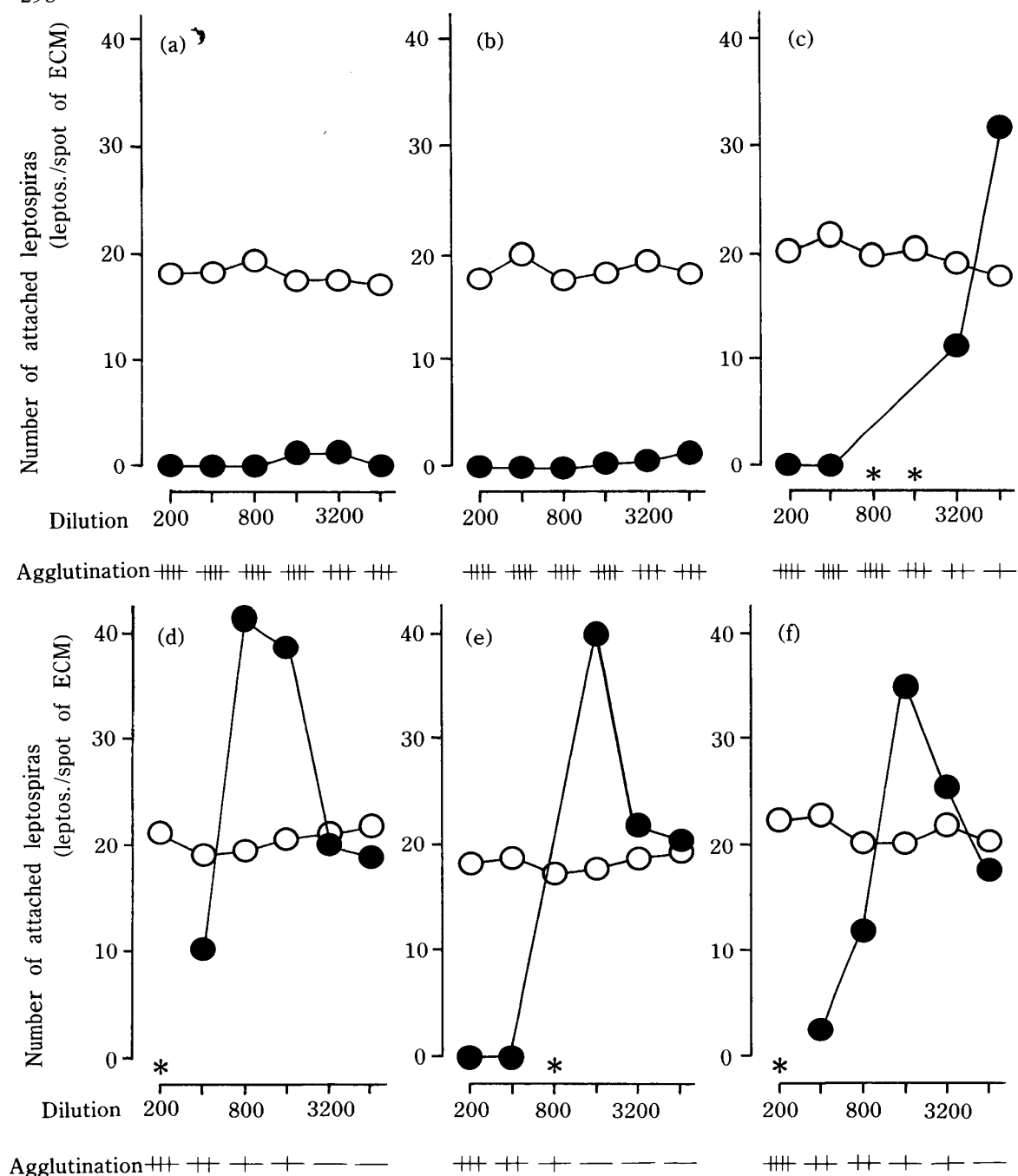


FIGURE 4 Attachment of antigenic variant and parent to ECM of mouse skin fibroblast cells in the presence of antiserum against parent. (a) Hebdomadis (parent) (b) HV(H19)-1 (c) HV(H1)-1 (d) HV(H16)-1 (e) HV(H14)-1 (f) HV(H9)-1 Symbols: (○) normal rabbit serum (●) anti-Hebdomadis antiserum. Degree of microscopic agglutination; ++++(100%) ++(75%) +(50%) +(25%) -(0%) \*indicates attachment of leptospires was undetectable because of many clumps of agglutinated leptospires.

## DISCUSSION

The antigenic variants of *L. interrogans* serovar *canicola* Moulton and *hebdomadis* Hebdomadis, which were isolated from each parental population by selection with monoclonal antibodies, demonstrated attachment to the cells and ECM in the presence of anti-parent antiserum. The serological behavior of the antigenic variants was completely different from that of the parent. The results clearly indicated that antigenic variants which were present in the population of *L. interrogans* attached to the cells and ECM, resisting the effect of anti-parent antiserum. Resistance of antigenic variants intrinsically present in the population of *L. interrogans* to the effect of anti-parent antiserum may be important from the viewpoint of prevention of leptospiral infection. It was reported that vaccine prepared with the parent protected the challenge of some antigenic variants, while the vaccine prepared with the variants did not protect the challenge of the parent.<sup>16)</sup> However, it is possible that vaccine prepared with the parent does not prevent infection of the variants as the anti-parent antiserum dose not inhibit attachment of the variants. This speculation is a subject of future interest.

The resistance of the variants to the effect of each anti-parent antiserum on the attachment was related to their serological remoteness from the parent. It is interesting that only the antigenic variants serologically remote from the parent could resist the pressure of anti-parent antiserum, but that those not remote from the parent could not.

The antigenic variants were found to attach to mouse skin fibroblast cells and ECM, and the number of attached leptospiaras was similar between the parents and variants. This is the first observation of the attachment of antigenic variants of leptospiaras. The results may suggest that antigenic variants intrinsically present in the population of *L. interrogans* are able to attach to host cells or tissues.

Avirulent strains attached only poorly to the established cell lines such as L929 and MDCK cells and ECM.<sup>7,14,15)</sup> In the present study, secondary mouse skin fibroblasts which attached more avirulent leptospiaras than to established cell lines did were used.

Vinh et al.<sup>15)</sup> demonstrated enhancement of leptospiral attachment by subagglutinating amounts of homologous antibody. In the present experiments, enhancement of attachment was observed at the dilution of antiserum showing weak agglutination. The reason for the enhancement of attachment is not clear.

Attachment of antigenic variants of leptospiaras to cells and ECM in the presence of anti-parent antiserum, as shown in the present study, deserves special attention from the viewpoint of the break of vaccination. More attention should be focused on antigenic variation of leptospiaras.

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