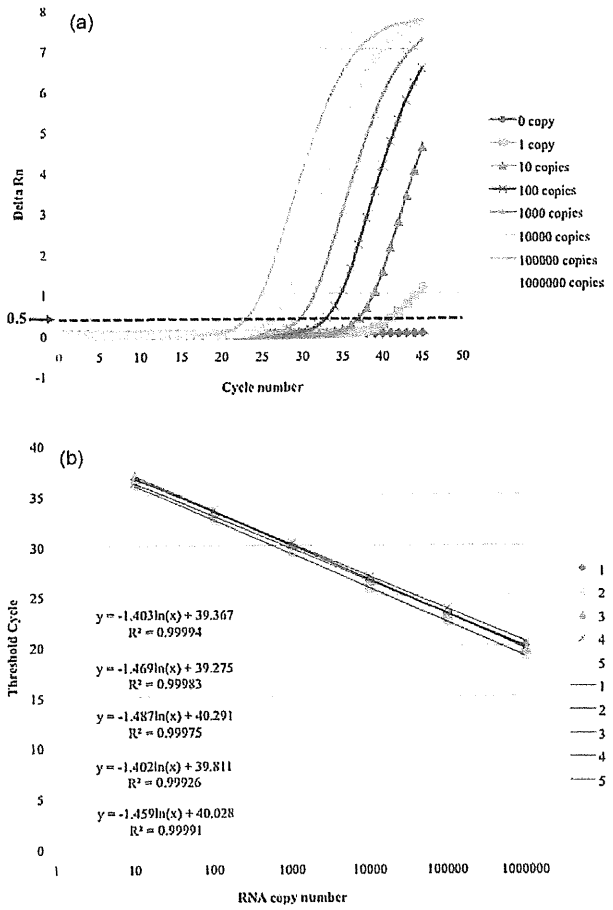

Fig. 1. Determination of the cut-off values (a) and standard curves (b). Ten-fold dilutions of standard RNA were used with the TaqMan assay. (a) Each sample was amplified in triplicate. The broken line indicates $\Delta R_n = 0.5$. (b) Five independent experiments were performed in triplicate. Each numerical expression represents a regression line, and R^2 indicates the coefficient of determination.

ing TaqMan primers and probes using Primer Express Software Ver. 3 (Applied Biosystems, Foster City, CA). The sequence of the probe was 5′-CCGTCGGCAGTTGG-3′ (encoding nt 93–106), that of the forward primer was 5′-CCTAHYCCCATGGAGAAACTCCT-3′ (nt 32–54), and that of the reverse primer was 5′-AACATCGCGCACTTCCCA-3′ (nt 143–160). The reporter probe was conjugated with 6-carboxyfluorescein and minor groove binder at the 5′ and 3′ termini, respectively.

To determine the cut-off values for the TaqMan assay, synthesized RNA in vitro (consisting of 1–782 nt of TO336 wt-type) was quantified by OD_{260}, and a 10-fold dilution series was prepared (from 2.0×10^5 to 2.0×10^{-1} copies/μl) and applied to the TaqMan assay (Fig. 1a). The assay was performed by using a TaqMan RNA-to-C_T 1-Step Kit (Applied Biosystems) in a total volume of 20 μl, which contained a final concentration of 900 nM sense and antisense primers, 250 nM probe, 1× TaqMan RT Enzyme Mix, and 1× TaqMan RT-PCR Mix. The kinetics of cDNA amplification were monitored using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) under the conditions of 48 °C for 15 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 s and 60 °C for 1 min. This assay was carried out in triplicate for each sample, including a no-template control. Test samples were considered positive if amplification with a threshold cycle (Ct) value <40 and ΔR_n signal >0.5 were seen in all of the triplicate reactions. Five distinct sets of 10-fold-diluted standard RNA samples (1.0×10^6 to 1 copies/reaction) were examined to estimate the dynamic range of this assay. Although above 10 copies/reaction were detected in all samples, 1 copy/reaction was detected in 47% of samples (Table 1). The standard curve from 1 to 1.0×10^6 copies/reaction did not satisfy a linear relationship of $R^2 > 0.99$ (data not shown); therefore, the data of one copy/reaction was omitted from the standard curve. Log-linear regression plots showed a strong linear relationship ($R^2 > 0.99$) between the log of the starting copy number (from 10 to 1.0×10^6 copies) and the Ct values (Fig. 1b). All standard curves showed a similar slope and intercept, and the reproducibility of the standard curve was reliable, at least in the range from 10 to 1.0×10^6 copies/reaction. These results indicate that the dynamic range of this assay was from 10 to at least 1.0×10^6 copies/reaction.

Thirteen RV genotypes have been recognized to date (WHO, 2007b), all belonging to only one serotype. These genotypes are classified into two clades: clade1 and clade 2. Clade 2 has not been reported in Japan, except for Rvi/OSAKA.JPN/11.07 (2B), isolated in 2007. Few studies using real-time PCR for detection of the RV genome have compared the sensitivity and specificity between the viral genotypes or strains using RNA extracted from viral stocks, although several investigators have used real-time PCR for RV diagnosis (Abernathy et al., 2009; Hübschen et al., 2008; Rajasundari et al., 2008; Zhao et al., 2006). To investigate the sensitivity and specificity of the TaqMan assay for various viruses, 10 RV strains consisting of five genotypes (1a, 1B, 2B, 1D and 1j), which included vaccine strains (Shishido and Ohtawara, 1976) that have been isolated, propagated, and titrated in our laboratory, were examined using this assay, and the results were compared with a conventional RT-PCR. The viral RNA was prepared from 140 μl of culture medium of RK-13 cells infected with each RV, using a QIAamp Viral RNA Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's protocol, extracted with 70 μl of elution buffer, and each 5 μl of eluted sample was subjected to the TaqMan assay and the conventional RT-PCR. The conventional RT-PCR conditions are described below.

For amplifying an 851-nt region in the E1 coding region (nt 8702–9553) using a primer set F1 (5′-CGACGCGGCCTGCTGGGGC) and R9 (5′-AGGTCTGCCGGGTCTCCGAC), RT-PCR was performed with One-Step RT-PCR Kit (QIAGEN) according to the manufacturer's protocol, except that the total reaction volume and concentration of the primers were altered to 25 μl and 0.3 μM, respectively. After carrying out the RT reaction for 30 min at 55 °C and denaturation for 15 min at 95 °C, the reaction mix-

Table 1
Detection limit of the TaqMan assay using a series of synthesized standard RNA.

Standard RNA (copies/reaction)	Number of positive samples/number of tested samples					Positive (%)
	1	2	3	4	5	
10^6	3/3	3/3	3/3	3/3	3/3	100%
10^5	3/3	3/3	3/3	3/3	3/3	100%
10^4	3/3	3/3	3/3	3/3	3/3	100%
10^3	3/3	3/3	3/3	3/3	3/3	100%
10^2	3/3	3/3	3/3	3/3	3/3	100%
10	3/3	3/3	3/3	3/3	3/3	100%
1	0/3	2/3	0/3	3/3	2/3	47%

tures were incubated for 35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 10 min. The RT-PCR product (0.5 μl) was used as a template for a nested PCR with 2 mM $MgCl_2$, 50 mM KCl, 25 mM TAPS buffer (pH 9.3), 200 μM sense (5′-CAGCACCCTCACAAGACCGTC-3′) and anti-sense (5′-CACAGCAGTGGTGTGTGTGCC-3′) primers, and 0.025 U Ex Taq polymerase (TaKaRa Bio, Shiga, Japan). The cycling conditions were as follows: 98 °C for 30 s, and 30 cycles of 98 °C for 10 s and 68 °C for 1 min. The PCR products were resolved on a 1% agarose gel and visualized by ethidium bromide staining. As a result, at least 1 pfu of RV could be detected in Rvi/OSAKA.JPN/11.07 (2B), SRL6.97 (1D), and Osaka' 94 (1D) strains by the TaqMan assay (Table 2), although a greater viral load was required in the other viral strains (from 10 to 100 pfu). On the other hand, at least 10 pfu of the virus was required for detection by the conventional RT-PCR, and for the Kagoshima' 04 strain (1j), as much as 1000 pfu of the virus was required. Thus, the novel real-time PCR had about 10-fold greater sensitivity compared with the conventional RT-PCR. Although the conventional RT-PCR amplified a relatively long region (851 bp), the sensitivity of this method was almost identical to that of another RT-PCR amplifying 481 bp (data not shown). Conventional RT-PCR amplifying the 851-nt sequencing region in the E1 coding region could be used for direct genotyping. Thus, the conventional RT-PCR is also thought to be useful for rapid genotyping. The sequences of the target region for the TaqMan assay were identical among genotype 1a strains (data not shown). The reason for the low sensitivity of the TaqMan assay for the TO336 wild-type and the Matsuura vaccine strains is unknown.

It is necessary for monitoring to confirm the morbidity of rubella precisely, and it is especially important to distinguish rubella from other infectious diseases. Clinical diagnosis of rubella is sometimes confused with measles, HHV-6, and parvovirus B19, because they have similar major symptoms to rubella (WHO, 2007a). Therefore, to exclude the possibility of false-positive results for other viruses, measles viral RNA (strains SA203, Yamagata, IC-B, YS-4, Edmonston, and Toyoshima, kindly provided by Dr. Seki, Department of Virology III, National Institute of Infectious Diseases), HHV-6 type A DNA (kindly provided by Dr. Inoue, Department of Virology I, National Institute of Infectious Diseases), and parvovirus B19 DNA (kindly provided by Dr. Okada, Department of Blood, National Institute of Infectious Diseases) were examined (Table 3). Measles virus strains used in this study included wild-type isolates with various genotypes that are found frequently in Japan or Asia (D5, D9, and H1) (CDC, 2005), a vaccine strain (A), and a laboratory strain (D3). Although the amount of template in the measles virus and parvovirus B19 was not known precisely, the samples were confirmed as positive by conventional RT-PCR or PCR-specific for each virus (data not shown). These viral RNAs or DNAs were not detected by the probe or primers used in this study.

It is difficult to obtain an adequate number of clinical samples from patients suspected of rubella infection in Japan, because the morbidity rate decreased significantly in recent years. Therefore, to mimic the detection of viral RNA extracted from clinical specimens, a spike test was performed as an alternative resource. Throat swabs from healthy donors collected in a Universal Viral Transport (UVT) medium (BD, Franklin Lakes, NJ) were added to 1, 10, 10^2, and 10^3 pfu of three viral strains, TO336 vaccine (1a), Osaka' 94 (1D), and Kagoshima' 04 (1j), and subjected to the TaqMan assay and the conventional RT-PCR. Ten pfu of all viruses could be detected by the TaqMan assay (Table 4). However, even 10^3 pfu of each virus could not be detected by the conventional RT-PCR. The TaqMan assay detected viral RNA from the spiked test samples to almost the same degree as with a viral culture medium. This showed that neither the RNA extraction step nor the TaqMan assay was affected by the presence of contaminants included in clinical specimens (i.e., throat swabs).

Table 2
Sensitivity of the TaqMan and conventional RT-PCR assay.

Strain	Genotype	Viral dose (pfu)											
		10^4		10^3		10^2		10^1		10^0		10^{-1}		10^{-2}	
		TaqMan	RT-PCR	TaqMan	RT-PCR	TaqMan	RT-PCR	TaqMan	RT-PCR	TaqMan	RT-PCR	TaqMan	RT-PCR	TaqMan	RT-PCR
TO336 wild	1a	27.5 ± 0.25	N/A	31.3 ± 0.30	+	35.4 ± 0.25	+	40.0<	−	40.0<	−	40.0<	N/A	40.0<	N/A
TO336 vaccine	1a	24.2 ± 0.26	N/A	28.2 ± 0.17	+	31.8 ± 0.26	+	35.4 ± 0.15	−	40.0<	−	40.0<	N/A	40.0<	N/A
Matsuura wild	1a	26.6 ± 0.34	N/A	30.3 ± 0.52	+	34.1 ± 0.51	+	37.5 ± 0.50	+	40.0<	−	40.0<	N/A	40.0<	N/A
Matsuura vaccine	1a	28.1 ± 0.48	N/A	31.6 ± 0.39	+	35.5 ± 0.53	+	40.0<	−	40.0<	−	40.0<	N/A	40.0<	N/A
SRL 8.79	1B	25.0 ± 0.94	N/A	28.6 ± 0.86	+	32.4 ± 0.79	+	36.0 ± 1.00	+	40.0<	−	40.0<	N/A	40.0<	N/A
Rvi/OSAKA.JPN/11.07	2B	22.1 ± 0.42	N/A	25.2 ± 0.29	+	28.9 ± 0.21	+	32.7 ± 0.39	+	36.2 ± 0.15	−	40.0<	N/A	40.0<	N/A
SRL 6.97	1D	24.4 ± 0.34	N/A	27.5 ± 0.24	+	31.0 ± 0.19	+	35.0 ± 0.08	−	38.8 ± 0.15	−	40.0<	N/A	40.0<	N/A
Osaka' 94	1D	23.7 ± 0.31	N/A	27.3 ± 0.22	+	31.0 ± 0.21	+	34.6 ± 0.35	+	38.5 ± 1.11	−	40.0<	N/A	40.0<	N/A
Miyazaki' 01	1j	25.3 ± 0.16	N/A	29.0 ± 0.19	+	32.9 ± 0.21	+	36.5 ± 0.22	+	40.0<	−	40.0<	N/A	40.0<	N/A
Kagoshima' 04	1j	26.3 ± 0.18	N/A	29.8 ± 0.19	+	33.5 ± 0.10	−	37.3 ± 0.41	−	40.0<	−	40.0<	N/A	40.0<	N/A

Table 3
Specificity of the TaqMan assay.

Virus			Amount of template	Ct	Result
RV					
	TO336 wild	1a	100 pfu	35.4 ± 0.25	Pos.
	TO336 vaccine	1a	100 pfu	31.8 ± 0.26	Pos.
	Matsuura wild	1a	100 pfu	34.1 ± 0.51	Pos.
	Matsuura vaccine	1a	100 pfu	35.5 ± 0.53	Pos.
	SRL 8.79	1B	100 pfu	32.4 ± 0.79	Pos.
	Rvi/OSAKA.JPN/11.07	2B	100 pfu	28.9 ± 0.21	Pos.
	SRL 6.97	1D	100 pfu	31.0 ± 0.19	Pos.
	Osaka' 94	1D	100 pfu	31.0 ± 0.21	Pos.
	Miyazaki' 01	1j	100 pfu	32.9 ± 0.21	Pos.
	Kagoshima' 04	1j	100 pfu	33.5 ± 0.10	Pos.
MV					
	SA203	D5	N/A	40.0<	Neg.
	Yamagata	D9	N/A	40.0<	Neg.
	IC-B	D3	N/A	40.0<	Neg.
	YS-4	H1	N/A	40.0<	Neg.
	Ed	A	N/A	40.0<	Neg.
	Toyoshima	A	N/A	40.0<	Neg.
Others					
	HHV-6 U1102	A	0.5 μg of DNA	40.0<	Neg.
	HHV-6 Z29	B	0.5 μg of DNA	40.0<	Neg.
	Parvovirus B19		N/A	40.0<	Neg.

Table 4
Spike test for detection of RV in throat swabs.

Strain	Genotype	Viral dose (pfu)			
		10^3		10^2	
		TaqMan	RT-PCR	TaqMan	RT-PCR
TO336 vaccine	1a	29.4 ± 0.13	–	33.1 ± 0.11	–
Osaka' 94	1D	27.8 ± 1.12	–	32.1 ± 1.33	–
Kagoshima' 04	1j	28.5 ± 1.12	–	32.7 ± 1.45	–

Table 5
Detection of RV in clinical specimens by TaqMan assay.

Individuals	Specimens	TaqMan #1	Ct	TaqMan #2	Ct	RT-PCR	Virus isolation
1	Oral fluid	Pos.	34.9	Pos.	35.7	Pos.	Pos.
2	Oral fluid	Pos.	39.4	Pos.	39.3	Neg.	Pos.
3	Oral fluid	Pos.	38.4	Pos.	37.9	Neg.	Pos.
4	Oral fluid	Pos.	36.1	Pos.	36.7	Pos.	Pos.
5	Oral fluid	Pos.	38.5	Pos.	39.1	Neg.	Pos.
6	Oral fluid	Pos.	31.6	Pos.	33.7	Neg.	Pos.

To compare the sensitivity of the novel TaqMan assay in clinical specimens with the conventional RT-PCR, six oral fluids collected from an outbreak of rubella in 2004 in Japan were tested using the TaqMan assay (TaqMan #1) and conventional RT-PCR. All samples were confirmed as positive by the conventional RT-PCR after passages in culture. As a result, all specimens (6/6) were positive using the TaqMan assay, although two of the six samples were positive using the conventional RT-PCR (Table 5). The same samples were also subjected to another TaqMan assay (TaqMan #2), which has been reported previously (Zhu et al., 2007; Abernathy et al., 2009). However, no amplification was detected both in the negative control and in the positive control using the TaqMan #2 method (data not shown). When the primers and probe were used with the same reagent in this study (TaqMan RNA-to-C_T 1-Step Kit), amplification signals were obtained. Under these conditions, all specimens (6/6) were also found to be positive, with similar Ct values to the TaqMan #1 method (Table 5). Although it was unclear why the primers and probe of TaqMan #2 method did not function under the assay conditions described previously, the primers and probe could function equally well as those of the novel TaqMan method (TaqMan #1) under the assay condition used in TaqMan #1 method. The TaqMan assay used in this study was more reliable, because it was confirmed that this assay could detect various viral strains sensitively and did not cross-react with other viral RNAs causing similar symptoms as rubella.

Detection of viral RNA in oral fluid seems to be more suitable for rapid diagnosis of rubella. WHO does not recommend viral genome detections for laboratory confirmation, probably due to the possibility of laboratory contamination and cross-contamination when performing RT-PCR followed by nested PCR. However, given the lower risk of contamination using real-time PCR and its higher sensitivity compared with conventional RT-PCR, the former technique is concluded to be more suitable for rapid diagnosis. The new real-time PCR assay described above was able to detect at least 10 copies of RV RNA and 1 pfu of virus. This TaqMan PCR assay is considered to be useful for rapid diagnosis and screening of rubella when used in conjunction with conventional RT-PCR.

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Ethics Committee approval for the collection of throat swabs from the authors for the use as controls was not required by the National Institute of Infectious Diseases.

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INFLAMMATORY DISORDERS

Rubella virus as a possible etiological agent of Fuchs heterochromic iridocyclitis

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Abstract
Background To determine whether rubella virus is involved in the pathogenesis of Fuchs heterochromic iridocyclitis (FHI).
Methods Fourteen patients (14 eyes) diagnosed with FHI based on characteristic ocular manifestations and eight control subjects were studied. Aqueous humor (AH) samples from 14 FHI patients and one vitreous sample from a FHI patient were analyzed for intraocular antibody production against rubella virus by calculation of the Goldmann–Witmer coefficient (GWC). Viral detection by nested polymerase chain reaction and isolation by culture in RK-13 cells were conducted in nine FHI patients. In addition to laboratory examinations, medical history of rubella virus vaccination was also obtained.
Results Ten patients with FHI examined showed intraocular synthesis of rubella virus antibodies (GWC>3). A high index of rubella virus antibody production was also found in the vitreous sample (GWC=30.6). GWC in all control subjects were below detectable level. The rubella genome was detected in two of nine patients, and rubella virus was isolated from one of nine patients with FHI. None of the patients with FHI had been vaccinated against rubella.
Conclusions Our laboratory data strongly suggest a relationship between FHI and rubella virus.

Keywords Fuchs heterochromic iridocyclitis · Rubella virus · Viral isolation · Vaccination

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Introduction

Fuchs heterochromic iridocyclitis (FHI) is an intraocular inflammatory disease that constitutes approximately 0.5% to 6.2% of all cases of uveitis [1, 2]. FHI usually occurs in only one eye. Common clinical manifestations are: (1) chronic low-grade iridocyclitis including keratic precipitates, (2) iris heterochromia, atrophy or both, (3) absence of synechiae, and (4) early cataract [3, 4]. Complications such as glaucoma and vitreous opacities have been reported in 14.8% of patients with FHI [5]. Patients may remain asymptomatic for years, and diagnosis is often made by a decrease in visual acuity secondary to cataract which was observed in 77.8% at presentation. Therefore, it is difficult to detect FHI at the early stage and to prevent disease progression.

The etiology of FHI remains unknown. Because of the association between FHI and Horner's syndrome, sympathetic nerve dysfunction was considered to be a cause of FHI [6]. Saari et al. [7] also reported vascular abnormality of the iris in patients with FHI, as demonstrated by fluorescein angiography. In addition to the organic abnormalities in patients, some infectious agents such as *Toxoplasma gondii* [8], herpes simplex virus (HSV) [9] and cytomegalovirus (CMV) [10], as well as auto-antigens [11, 12] have been proposed as possible causes of FHI. Recently, several studies have implicated rubella virus infection as a possible etiological agent of FHI [13–16].

Table 1 Demographic and clinical background of 14 patients studied

Case	Age	Gender	Medical history	
			Rubella infection	Rubella vaccination
1	55	male	+	–
2	50	female	+	–
3	47	female	–	–
4	60	male	+	–
5	41	male	+	–
6	50	male	+	–
7	39	male	+	–
8	26	female	–	not known
9	36	female	+	–
10	27	male	+	–
11	51	male	not known	–
12	62	female	+	–
13	39	female	+	–
14	54	female	+	–

However, it is suggested that intraocular presence of rubella virus is not necessary for the development of FHI [13, 16].

The objectives of the present study were to verify the relationship between rubella virus infection and FHI by examining intraocular antibody production and detecting viral RNA by polymerase chain reaction (PCR), and to attempt to isolate rubella virus from aqueous humor of FHI patients to confirm the intraocular presence of the virus.

Material and methods

Fourteen Japanese patients (14 eyes) with FHI who attended the uveitis clinic of Tokyo Medical University Hospital between 2006 and 2009 were enrolled in this retrospective review. The study was approved by the institutional review board.

Diagnosis of FHI was based on characteristic ocular manifestations including chronic anterior intraocular inflammation, keratic precipitate, absence of posterior synechiae, heterochromia or anterior stromal iris atrophy, and secondary cataract. The demographics and clinical background of the patients with FHI are shown in Table 1.

As controls, eight Japanese patients with other types of uveitis comprising sarcoidosis (one), Posner–Schlossman syndrome (one), herpetic iritis (one), Behçet disease (one) and unclassified intraocular inflammation (four) were selected. The diseases of the control patients were clearly differentiated from FHI by clinical manifestations and laboratory studies. Demographics of FHI patients and controls are listed in Table 2.

Aqueous humor (AH) samples were obtained from all FHI patients during surgery for secondary cataract or secondary glaucoma. All patients with FHI had no or low-grade ocular inflammatory activity at the time of sample collection. One vitreous humor (VH) sample was also obtained during vitrectomy for vitreous opacity. Rubella antibody titers in intraocular fluid (AH and VH) and serum samples from 14 FHI patients were determined by fluorescent antibody (FA) and enzyme immunoassay (EIA) techniques. Paired intraocular fluid and serum samples from each patient were tested at the same time. The Goldmann–Witmer coefficient (GWC) was calculated as follows: quantity of rubella virus-specific IgG/total IgG in intraocular fluid divided by rubella virus-specific IgG/total IgG in serum. A GWC value exceeding 3 was considered to indicate local antibody production, as described previously [14].

Rubella virus detection and isolation were conducted using AH samples from the nine most recent FHI patients (cases 6 to 14). For rubella virus isolation, RK-13 cells were inoculated with AH and incubated, and the cell cultures were serially passaged. The RK-13 cells were lysed by rapid freezing and thawing, and the lysate obtained was used for reverse transcription-polymerase chain reaction (RT-PCR). For rubella virus detection from ocular samples, total RNA was extracted from the samples using the High Pure Viral RNA kit (Roche Diagnostics, UK). For detecting rubella viral RNA, two nested RT-PCR were conducted for two parts of the E1 gene, designated as E1-2 region (466 bp) and E1-3 region (423 bp). The primer designs used for RT-PCR were listed in Table 3. The first round RT-PCR was performed as follows: 50°C for 30 min, followed by 95°C for 5 min, then 40 cycles of 90°C for 30 s, 61°C for 30 sand 72°C for 1 min., and 72°C for 5 min. For the nested PCR, 25 cycles of 98°C for 10 s, 59°C for 30 s (E1-2 region) or 66°C for 30 s (E1-3 region) and 72°C for 45 s

Table 2 Demographics and clinical characteristics of FHI and control patients

RV: rubella virus, [a]: Goldmann–Witmer coefficient could not be calculated in four cases due to insufficient ocular sample for IgG measurement

*: $p<0.01$: Fisher's exact test

	FHI	Control
Cases	14	8
Male	7 (50%)	3 (38%)
Female	7 (50%)	5 (62 %)
Age (years)	45.5 (27–62)	69.5 (45–85)
Intraocular antibody production against RV	10/10[a] (100%)	0/8 (0%)*

Table 3 Primers for rubella virus RT-PCR

Region	PCR	Primer	Sequence
E1-2	First	E1-2F	5′- AGCGACGCGGCCTGCTGGG
		E1-2R	5′- CCAGCGCGTATGTGG AGTCC
	Nested	E1-6F	5′- ACACCGTGATGAGCGTGTTC
		E1-10R	5′- ATGT GGAGTCCGCACTTGCG
E1-3	First	E1-7F	5′- TTGTGGGGGCCACGCCAGAG
		E1-12R	5′- TGTGTGCCATACACCA CGCC
	Nested	E1-3F	5′- CGGCGAGGTGTGGGTCACGC
		E1-3R	5′- ACCCGCGCGCTCGCGCGATC

were conducted. The PCR products were confirmed by electrophoresis in 1.5% agarose gel.

In addition to laboratory examinations, medical history related to exposure to rubella (vaccination or infection) was also obtained.

Results

Antibody titer and GWC for rubella virus

We attempted to measure rubella antibody titers and determine GWC using paired AH and serum samples collected from 14 patients with clinically definite FHI. Ten FHI patients demonstrated intraocular synthesis of rubella virus antibodies (GWC>3) with median GWC of 45.1 (total range 5.7–186.1) (Fig. 1). In the remaining four patients with FHI (cases 11 to 14), total IgG in AH could not be measured because of inadequate AH samples, and therefore GWC could not be determined, although rubella antibody was positive in their AH samples (case 11: 32.5 IU, case 12: 130 IU, case 13: 20 IU, case 14: 28.5 IU). None of the control subjects had antibodies against rubella in AH samples and GWC values were below detectable level. The rates of intraocular antibody synthesis were significantly different ($P<0.01$; Fisher's exact test) between patients with FHI (ten of ten) and control patients (none of eight) (Table 2).

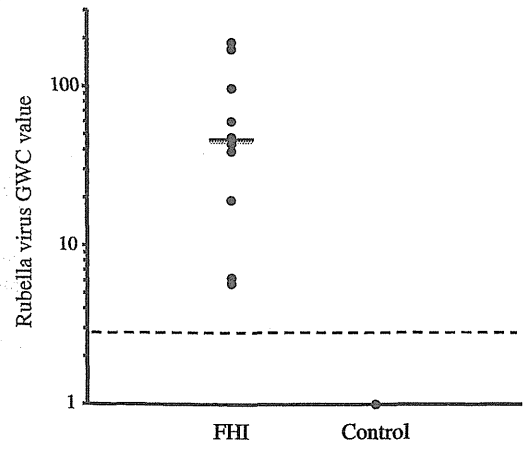

Fig. 1 Evaluation of the rubella virus Goldmann–Witmer coefficient (GWC) values of ten patients with FHI and eight control patients. The median value of FHI patients is indicated by a *horizontal black line*. The threshold GWC value 3 is indicated by the *dashed line*. The GWC value is presented in a logarithmic scale

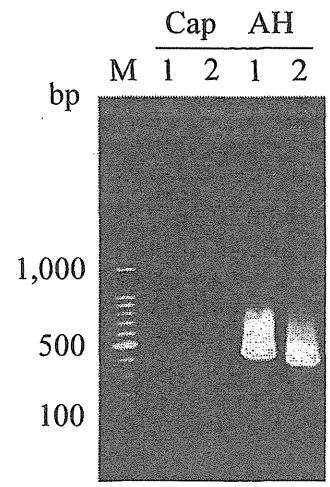

Fig. 2 Representative results of the detection of rubella virus gene from ocular samples of case 6 using two nested RT-PCR. Lanes *1 and 2* indicate the PCR products of E1-2 and E1-3 regions, respectively. Lane *M* shows the 100 bp DNA ladder markers. *Cap*; lens anterior capsule, *AH*; aqueous humor. Note that the PCR products from AH are positive for rubella virus

The vitreous sample from one FHI patient (case 6) was also examined. The rubella antibody titer in the VH sample was ×160, and total IgG was 14.9 mg/dl. The GWC of VH was high (GWC=30.6).

Rubella virus gene detection from AH and lens anterior capsule

Using RT-PCR, the rubella genome was detected in two of nine AH samples from FHI patients (cases 6 and 7). Virus detection was confirmed by two primer sets targeting the

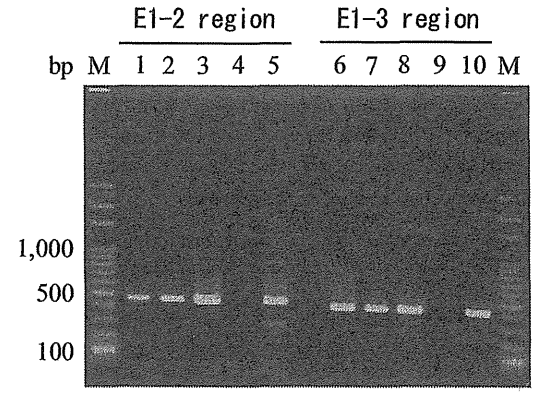

Fig. 3 Detection of rubella virus gene using RT-PCR from different passages of RK-13 cells inoculated with aqueous humor sample collected from case 7. Lanes 1-5 show the PCR results of E1-2 region, and lanes 6-10 show the PCR results of E1-3 region. Lanes 1-3 and 6-8 are the results from passages 1 to 3, respectively, of RK-13 cells inoculated with AH. Lanes 4 and 9 indicate negative control and lanes 5 and 10 indicate positive control. Lane Ms are 100 bp DNA ladder markers. Note that the PCR products from the 3rd passages of RK-13 cell are positive for rubella virus

E1 gene of rubella virus. The representative data of RT-PCR are shown in Fig. 2.

Rubella virus isolation from AH

Rubella virus isolation was attempted in nine patients with FHI (Cases 6 to 14). Rubella virus was isolated from one patient with FHI (case 7). The results of RT-PCR obtained from lysates of various passages of RK-13 cells are shown in Fig. 3.

Medical history of rubella vaccination and infection

Fourteen patients with FHI were questioned for a medical history of rubella vaccination and infection (Table 1). None of the patients with FHI had been vaccinated against rubella, although one patient was uncertain. A previous medical history of rubella infection was confirmed in 11 cases.

Discussion

Many previous reports have speculated the etiology of FHI, but recent reports of the relationship between rubella virus and FHI have renewed the interest. Quentin et al. [13] demonstrated the existence of rubella virus in AH by GWC determination and PCR assay, and de Groot-Mijnes et al. [14] confirmed the presence of rubella infection by calculating GWC. In this study, we also demonstrated rubella infection in AH and VH by GWC determination and PCR assay. The epidemiologic observation revealed reduced incidence of FHI following the introduction of vaccination against rubella virus [15]. None of our patients with FHI had received vaccination against rubella virus. Together with previous reports, these results confirmed the relationship between FHI and rubella virus.

With regard to the diagnosis of FHI, Ruokonen et al. [16] reported the usefulness of determining intraocular antibody production rather than detecting the virus gene by RT-PCR. In their report, intraocular antibody production against rubella virus was found in all cases, whereas only two of 20 cases had positive results for PCR. In our study, the rate of rubella antibody proportion was 10/10 and that of virus gene detection by PCR was 2/9, and these findings agree with Ruokonen's report.

On the other hand, rubella virus was isolated from the AH of one FHI patient in this study. Except with the congenital rubella syndrome, rubella virus is transmitted by the respiratory route, and replicates in the nasopharynx and lymph nodes and then spreads throughout the body causing fever and rash [17]. The general perception is that there is no carrier state and the reservoir exists only in active human cases. Therefore, it is unclear whether the virus detected and isolated from aqueous humor in the FHI patient was from acute infection, re-infection or re-activation of latent virus. In the case of congenital rubella syndrome, rubella virus may persist in the lens for many years [18]. We also tried to detect rubella virus from the lens anterior capsule in case 6 using RT-PCR, but the result was negative.

Since rubella virus has not been hitherto isolated from intraocular fluid of FHI patients, the genetic characters of rubella virus associated with FHI are unknown. In Japan, no nationwide epidemics of rubella have been documented since 1992 [19], but small outbreaks in local areas have been observed, and the virus genotypes isolated in various outbreaks were different [20]. By studying the genealogical background of rubella virus strains isolated from patients with FHI, it may be possible to determine the time of infection and the strain(s) with a predilection to induce FHI. Moreover, antigen-specific immune reaction has been suspected to play a role in the pathogenesis of FHI, based on the results of restriction of infiltrated T cells [21] and polymorphisms of cytotoxic T cell antigen 4 [22]. Analysis of the specific genetic modification of the rubella virus may clarify the characteristics and the tropisms of the virus. Efforts to isolate and characterize the virus from intraocular sites will elucidate the pathomechanism of FHI. Further investigations are warranted.

In summary, isolation of rubella virus from intraocular specimens of FHI patients is difficult. To the best of our knowledge, this is the first report of isolation of rubella virus from the aqueous humor of a patient with FHI. Intraocular existence of rubella virus in an FHI patient is a very interesting finding when considering the mechanism of FHI development. Although the number of samples examined in this study was small, the results confirm a relationship between rubella virus and FHI. Further investigations, especially in the isolation and characterization of rubella virus strains associated with FHI, are warranted.

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Original Article

Reevaluation of Laboratory Methods for Diagnosis of Measles

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SUMMARY: The purpose of this study is to reevaluate the sensitivities of different methods used in the diagnosis of measles including virus isolation, RT-PCR, and measurement of IgM. Sixty-three throat swabs, 84 peripheral blood mononuclear cell (PBMC) samples, and 85 plasma samples were collected from 85 cases of suspected measles. The sensitivity of virus isolation using throat swabs and PBMC in comparison with RT-PCR was 58.1 and 93.5%, respectively. We defined laboratory-confirmed cases as those in which at least one of the methods was positive. The percentage of positive results by the different methods was compared among 49 laboratory-confirmed cases. The percentage of positive results from PBMC by RT-PCR and virus isolation was 100 and 91.7%, respectively. The percentage of positive results from throat swabs by RT-PCR and virus isolation was 91.2 and 52.8%, respectively. The percentage of IgM positive (79.6%) was significantly lower than that of PBMC by RT-PCR. Ten of 27 plasma samples collected within 5 days of the onset of fever were IgM negative. In contrast, all of the 21 plasma samples collected 6 days after the onset of fever were IgM positive. In conclusion, the detection of measles virus RNA in PBMC by RT-PCR was the most effective method for diagnosis of measles.

INTRODUCTION

Measles is a highly infectious respiratory virus infection, with typical symptoms that include maculopapular rash, fever, cough, coryza, and conjunctivitis (1). The introduction of the live attenuated measles virus (MV) vaccine has decreased the frequency of measles outbreaks. As the prevalence of measles has declined, laboratory confirmation has become increasingly important (2,3). The detection of MV-specific IgM antibodies is recommended as the standard method for laboratory diagnosis by the World Health Organization (WHO) (4) and is used in countries throughout the world. However, the level of IgM may be low or absent in patients sampled in the early stage of infection (5). Virus isolation is widely used as a valuable diagnostic method. The detection of MV RNA by reverse transcription-polymerase chain reaction (RT-PCR) has been shown to be very effective for the diagnosis of measles (6,7). Throat swabs and peripheral blood mononuclear cells (PBMC) are used for virus isolation and RT-PCR (8). The detection of MV depends on the time of sample collection after the onset of symptoms. Therefore, to determine the sensitivity of different methods requires the comparison of the number of days that have elapsed since the onset of fever. The purpose of this study is to compare the percentage of MV-positive results by different methods including virus isolation, RT-PCR, and the measurement of IgM for diagnosis of measles.

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MATERIALS AND METHODS

Clinical samples: From May 2007 to August 2008, a total of 85 cases were reported to the Kobe Institute of Health as measles based on the typical symptoms of measles. The median age of patients was 15 years and patients' ages ranged from 0 to 44 years. Throat swabs and peripheral blood were collected from 0 to 14 days after the onset of fever. Informed consent was obtained from patients or their guardians. Sixty-three throat swabs, 84 PBMC samples, and 85 plasma samples were collected from 85 cases. Both virus isolation and RT-PCR were performed on 58 throat swabs and 54 PBMC samples. The samples were kept at 4°C until inoculation for virus isolation. The throat swabs were eluted into 2 ml of virus transport medium (bovine serum albumin, penicillin/streptomycin, amphotericin B in Dulbecco's modified Eagle medium) and centrifuged at 3,000 rpm for 10 min and filtered through 0.45 μm filter membrane. The throat swabs for RNA extraction were stored at −80°C until use. Two milliliters of peripheral blood was treated with EDTA, and plasma was collected after centrifugation at 3,000 rpm for 10 min. PBMC were separated by Ficoll-Hypaque graduation centrifugation. PBMC used for RNA extraction were resuspended into 1 ml of cellbanker +1 (Zenoaq, Fukushima, Japan) and stored at −80°C until use.

Virus isolation: Throat swabs and PBMC were inoculated with B95a cells and cultured at 37°C. When multinucleated giant cells were observed, MV isolation was confirmed by an indirect immunofluorescent test using an antibody specific to MV (9).

RNA extraction: RNA was extracted from 140 μl of throat swabs using the QIAamp Viral RNA Mini Kit (Qiagen, Tokyo, Japan) and from PBMC with the

QIAamp RNA Blood Mini Kit (Qiagen). RNA was resuspended in RNAase free H_2O or AVE buffer and stored at −80°C.

Nested RT-PCR: The primer sets for the nested RT-PCR were recommended by the National Institute of Infectious Diseases of Japan (10,11). Each procedure including RNA extraction, first and nested RT-PCR, and agarose gel electrophoresis was performed in separate laboratories to avoid laboratory contamination. The MV RNA was first converted to cDNA using PrimerScript™ RT Reagent Kit (Takara, Otsu, Japan). Reverse transcription was performed at 37°C for 15 min. The first and nested PCR were performed using PerfectShot™ Ex Taq (Takara). The 50 μl-reaction mixture used for the first PCR contained 20 pmol of each primer. The first PCR was performed with primers specific for the H gene (forward, AACGGATGAT CCAGTGATAG and reverse, TTGAATCTCGGTAT CCACTC) and involved 30 cycles of 98°C for 10 s, 53°C for 30 s, and 72°C for 1 min. The nested PCR was performed with the following primer set: forward, TACCTCTCATCTCACAGAGG and reverse, CAC CTAAGGCTAGGTTCTTC. The 50 μl-reaction mixture used for the nested PCR contained 20 pmol of each primer. Five microliters of the first round of PCR products was amplified. Amplification was performed via 30 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1 min.

Anti-MV IgM antibodies: MV-specific IgM antibodies in plasma were assayed using Measles IgM-EIA (Denka Seiken, Tokyo, Japan).

Statistical analysis: The percentage of IgM-positive results was compared with virus isolation and RT-PCR using the chi-square test. P values of <0.05 were considered to be statistically significant.

RESULTS

Table 1 shows the comparison of RT-PCR with virus isolation. Of the 58 throat swabs, 18 samples were virus isolation positive and 40 samples were virus isolation negative. Of the 40 virus isolation-negative throat swabs, 13 throat swabs were found to be positive by RT-PCR, indicating that the sensitivity of virus isolation using throat swabs was 58.1%. Of the 54 PBMC samples, 29 samples were virus isolation positive and 25 samples were virus isolation negative. Of the 25 virus isolation-negative throat swabs, 2 throat swabs were found to be positive by RT-PCR, indicating that the sensitivity of virus isolation using PBMC was 93.5%. All virus isolation-positive samples from throat swabs and PBMC were found to be positive by RT-PCR, indicating that the specificity of virus isolation was 100% when compared with RT-PCR. Forty-nine cases were diagnosed as measles on the basis that at least one of the methods showed a positive result.

The onset day was not known in 1 of 49 cases. Table 2 shows the detection of MV in throat swabs and PBMC in accordance with the sampling time in days after the onset of fever among 48 laboratory-confirmed measles cases. All throat swabs and PBMC collected from 4 to 5 days after the onset of fever were found to be MV positive by either virus isolation or RT-PCR. The MV-positive ratio by virus isolation was lower in throat swabs

Table 1. Comparison of RT-PCR with virus isolation

Throat swab

RT-PCR	Virus isolation		Total
	Positive	Negative	
Positive	18	13	31
Negative	0	27	27
Total	18	40	58

PBMC

RT-PCR	Virus isolation		Total
	Positive	Negative	
Positive	29	2	31
Negative	0	23	23
Total	29	25	54

Table 2. Detection of measles virus by days after fever onset

Throat swab

Days after fever onset	Virus isolation			RT-PCR		
	Positive	No. of test	% of positive sample	Positive	No. of test	% of positive sample
0–3	2	6	33.3	4	6	66.7
4–5	11	11	100	10	10	100
6–12	5	18	27.8	16	17	94.1

PBMC

Days after fever onset	Virus isolation			RT-PCR		
	Positive	No. of test	% of positive sample	Positive	No. of test	% of positive sample
0–3	5	7	71.4	5	5	100
4–5	19	19	100	12	12	100
6–12	19	21	90.5	13	13	100

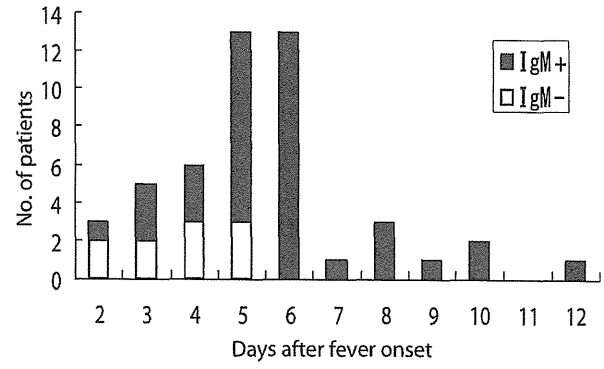

Fig. 1. The results of IgM positivity in plasma from measles patients by days after fever onset. Forty-eight plasma samples were collected from 48 cases with laboratory-confirmed measles cases.

Table 3. Comparison of diagnostic methods in measles cases

Sample	Method	No. of test	No. of positive sample	% of positive sample
PBMC	RT-PCR	31	31	100
	Isolation	48	44	91.7
Throat swab	RT-PCR	34	31	91.2
	Isolation	36	19	52.8[1)]
Plasma	IgM	49	39	79.6[2)]

49 cases were laboratory-confirmed measles cases. Number of test indicates the sample numbers tested by each method from 49 laboratory-confirmed measles cases.
1): P value, 0.0004 compared with throat swabs by RT-PCR.
2): P value: 0.007 compared with PBMC by RT-PCR.

collected from 0–3 days or 6–12 days after the onset of fever than those collected 4–5 days after the onset of fever. Thirty-nine of the plasma samples were IgM positive. Ten IgM-negative cases involving throat swab or PBMC were found to be positive by virus isolation or RT-PCR. All plasma samples collected 6 days after the onset of fever were IgM positive. In contrast, plasma collected within 5 days of the onset of fever showed both IgM positive and negative results (Fig. 1). Table 3 shows the percentage of positive samples according to diagnostic methods used in 49 laboratory-confirmed measles cases. One hundred percent and 91.7% of PBMC samples were found to be positive by RT-PCR and virus isolation, respectively. In contrast, the percentage of positive results in the throat swabs by virus isolation was significantly lower when compared with RT-PCR. The percentage of IgM-positive samples was significantly lower than that of PBMC by RT-PCR.

DISCUSSION

In this study, the numbers of samples used for virus isolation, RT-PCR, and measurement of IgM were different because throat swabs and peripheral blood were not collected from all patients. All laboratory-confirmed cases had symptoms such as fever, maculopapular rash, cough, coryza, and/or conjunctivitis. We consider that throat swabs and peripheral blood samples were collected from patients with cases of measles. We demonstrated that the sensitivity of RT-PCR was higher than that of virus isolation and that the virus isolation-positive ratio from throat swabs was lower than that obtained from PBMC. We have reported that the minimum amount of MV RNA detectable by SYBR Green real-time RT-PCR is 10 copies and that the sensitivity of nested RT-PCR is similar to that of SYBR Green RT-PCR (11). We have also previously detected MV RNA from virus stock containing 1 PUF/0.1 ml samples and virus isolation-negative throat swabs (11). It has been reported that MV isolation is less reliable as a diagnostic tool than serology or RT-PCR (7). The RT-PCR is able to detect MV RNA from samples containing noninfectious MV. We showed that the MV isolation rate from PBMC was higher than that from throat swabs. We kept the samples at 4°C and avoided freeze-thawing procedures. The lower virus isolation rate in throat swabs may have been caused by the loss of MV infectivity during handling or transportation. In addition, throat swabs were centrifuged and filtered through a membrane to remove bacteria. Therefore, the cell-associated MV in throat swabs might have been removed by the centrifugation or filtration before inoculation to B95a cells. There is no significant difference between the percentage of MV detection in PBMC by virus isolation and RT-PCR. Lymphocytes and monocytes associated MV is able to be isolated from B95a cells by cell-to-cell spreading. In this study, MV was detected in throat swabs and PBMC from all cases by either virus isolation or RT-PCR 4 to 5 days after the onset of fever. The virus load is considered to be greatest in this phase. The detection rate of MV in throat swabs decreased 6–12 days after fever onset. The infectivity of MV decreases after the appearance of a rash due to an increase in the level of IgG antibody. Therefore, the timing of specimen collection after the onset of fever can affect the detection rate of MV.

WHO recommends the detection of MV-specific IgM antibodies as the reference standard for laboratory diagnosis. It has been reported that the detection of measles-specific IgM is the standard test for laboratory diagnosis of measles (4). Single serum samples can be used to diagnose measles cases if collected between 72 h and 4 weeks after rash onset using an IgM capture enzyme immunoassay (EIA) (5). The negative result for IgM in the early stage of infection does not always imply negativity of MV infection. In this study, the measurement of IgM antibodies was insufficient to diagnose measles within 5 days of the onset of fever. WHO recommends that laboratories request a second sample for repeat IgM testing (4). In this study, we used throat swabs and peripheral blood as diagnostic samples. It has been reported that MV was isolated from urine in patients with measles for up to 5 days (12) and MV RNA was detected in urine from vaccine recipients (13). Urine samples are useful for diagnosis of measles because they are easy to collect. Therefore, a comparison of sensitivity and specificity using urine samples, throat swabs, and PBMC is necessary.

In conclusion, RT-PCR of PBMC is the most effective diagnostic method for the diagnosis of measles.

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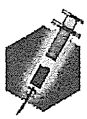
3. ワクチンを考えるうえで必要な臨床検査の知識
—抗体および抗体検査の意義と問題点

駒瀬勝啓[1)]

SUMMARY
抗体はB細胞から産生され，獲得免疫系の液性免疫では中心的な役割をもつ分子である．抗体の存在や抗体量を測定する抗体検査は，感受性調査，感染症の診断，ワクチンの有効性の検証などに用いられ，感染症対策に重要な役割を担っている．しかし，特に感染症の診断においては，測定法，検体の採取時期などから測定結果が必ずしも正しい診断につながらないことがある．本稿は抗体の概要を含めて，ウイルス感染症における抗体測定の意義，問題点などについて概説する．

〔臨床検査　54：1230-1238，2010〕

KEYWORDS
抗体，抗体検査，診断，感受性調査



免疫系と抗体

1．免疫系

地球上には多くのウイルス，細菌，カビ，寄生虫などの感染性微生物が存在する．これらの微生物がほかの生物に侵入し，そこである一定数以上に増殖すれば，生物の恒常性(ホメオスタシス)に直接あるいは間接的に影響を与え病気(感染症)となる．また，死に至らしめることもある．しかし多くの健常な生物は，常に微生物に曝露されながらも必ずしも病気にならないし，また，たとえ発病しても比較的短期間に治癒する場合が多い．これは生体が微生物を排除する免疫系を保持しているからである．免疫系は生体内に侵入してきた微生物を自分とは異なる分子(非自己)として認識し排除するシステムであり，その機構から非特異的に働く自然免疫系と抗原特異的に働く獲得免疫系に分かれる(表1)．

自然免疫系は生まれながらに備わっている免疫系で，いかなる微生物の侵入に対しても機械的かつ即座に作動する．自然免疫系では主に白血球のうち細菌や真菌を貪食する貪食細胞(好中球，単球，マクロファージなど)やウイルス感染細胞を破壊するナチュラルキラー(NK)細胞が働く．これらは微生物の侵入した局所へ遊走し微生物の排除に当たる．血液中の補体，皮膚や粘膜，繊毛のような微生物の感染に物理的に障害となるもの，胃酸，粘膜上のリゾチームなどの抗菌活性をもつ酵素，ウイルス感染細胞から産生されるインターフェロン(IFN)なども自然免疫系の一部である．

一方，自然免疫系だけでは微生物を排除できない場合には抗原特異的に反応するリンパ球による免疫応答である獲得免疫系がより強力に微生物を排除するように働く．抗原特異的とは，例えば麻疹ウイルスに対する免疫反応は次に麻疹ウイルスが侵入してきたときには機能するが，風疹ウイルスなどの感染には効果をあらわさないということである．また，獲得免疫系には，感染症に一度罹ったら2度罹らないようにする免疫記憶の機構も含まれる．

獲得免疫系の主役であるリンパ球は，白血球の一部であり骨髄中の造血幹細胞に由来する．リンパ球は骨髄で産生された後，胸腺で分化し細胞性免疫を司るTリンパ球(T細胞)と，骨髄で分化後，末端のリンパ節や脾臓などの2次リンパ組織に移動し液性免疫を司るBリンパ球(B細胞)に

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表1　自然免疫と獲得免疫の特徴

	自然免疫	獲得免疫
応答の特異性	なし	抗原特異的な応答
応答までの時間	短時間(即時)	比較的長い
免疫記憶	なし	あり
関与する可溶性因子	リゾチーム，補体，インターフェロンなど	抗体，サイトカイン
関与する主な細胞	貪食細胞(好中球，単球，マクロファージ)，ナチュラルキラー細胞	B細胞(液性免疫) T細胞(細胞性免疫)
生物界での分布	ほぼすべての生物	顎を持った脊椎動物以上

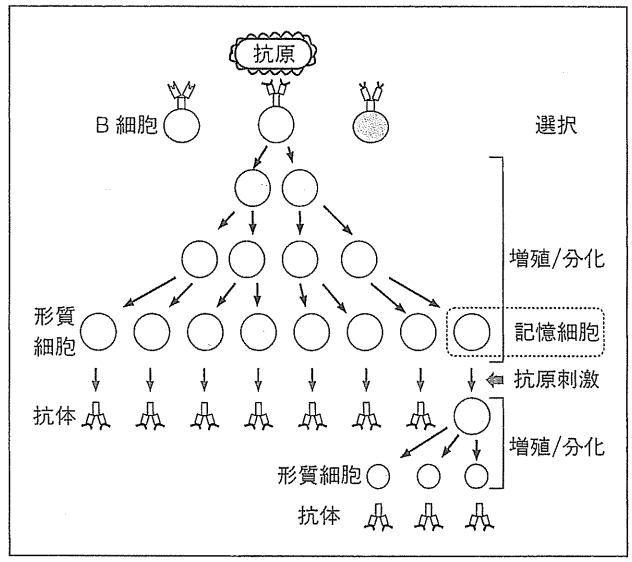


図1　B細胞が選択され抗原特異的抗体が産生されるまで

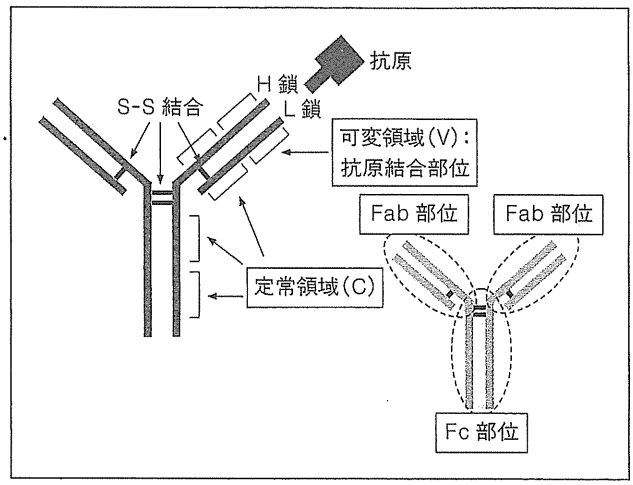


図2　抗体の構造

分かれる．細胞性免疫では，T細胞が様々なサイトカインを分泌して免疫系を制御するヘルパーT細胞や感染細胞を障害する細胞障害性T細胞などに分化し，微生物を排除する．液性免疫では抗原の刺激を受けたB細胞が分泌する，抗原特異的に結合する抗体(免疫グロブリンとも呼ばれる)が微生物の排除に働く．抗原による刺激を受けたB細胞，T細胞の一部は微生物を取り除いた後も免疫記憶細胞として生体に長期間生存し，再度同じ微生物の侵入を受けたとき，即座に活性化され微生物の再感染を阻止する．

2．抗体

1）抗原特異性の発現

抗体はB細胞から産生される抗原特異的に働く糖蛋白質である．この特異性の発現はB細胞の持つ抗原受容体(レセプター)による．B細胞は発生，分化の過程で抗原を認識するレセプターを細胞表面に発現する．そしてレセプターの構造は少しずつ異なり，認識できる抗原が異なる．つまり異なる抗原を認識するレセプターを細胞表面にもつ，無数のB細胞の集団が二次リンパ組織に準備されている状態にある．そこに微生物(抗原)が侵入すると，抗原を認識(抗原と結合)できるレセプターをもったB細胞のみが活性化され，増殖，分化し形質細胞となる．形質細胞はレセプターとほぼ同じ構造をもつ，抗原に特異的な抗体(免疫グロブリン)を血液中に分泌する．このように血中には感染した微生物に特異的な抗体の量が増加する．血中に存在する抗体の量を抗体価という．また免疫反応が収まった後も増殖した一部の細胞は記憶細胞として残り，再度同じ微生物の感染を受けたとき，即座に反応し抗体を量産する(図1)．

2）抗体の構造と機能

抗体にはB細胞の表面に存在し抗原レセプターとして働く膜結合型抗体と，分泌され血液やリンパ液中に存在する分泌型抗体がある．抗体の基本構造はY字型で，2本のH鎖(heavy chain)と2本のL鎖(light chain)の4本鎖構造からなり，H鎖同士とH鎖とL鎖はS-S結合で連結している(図2)．また，抗体をパパインという酵素で切断すると，2つのFab部分とFc部分に分解される．Fab部分のN末端には抗原と結合する機能があり，Fc部分は白血球やマクロファージ

表2　各クラスの抗体の特徴

	IgM	IgD	IgG	IgA	IgE
分子量(万)	97	18	16	16 (分泌時：39)	20
形	5量体	単量体	単量体	2量体 (分泌時)	単量体
Ig中の比率 (%)	10	<1	70～75	15～20	<1
半減期(日)	5	3	25	2	6
補体活性化	＋＋＋	－	＋＋	－	－
貪食細胞との結合能	＋	－	＋	－	－
胎盤通過	通過しない	通過しない	通過可能	通過しない	通過しない
エフェクター作用	補体の活性化 抗原の凝集	B細胞抗原受容体	中和，オプソニン化，補体の活性化	中和(粘膜上) 補体の活性化	肥満細胞の感作，好酸球の誘因
特徴	免疫反応の最初に出現，数か月で消失		比較的長期間存在，移行抗体	粘膜面に分泌	アレルギー反応，寄生虫排除に関与

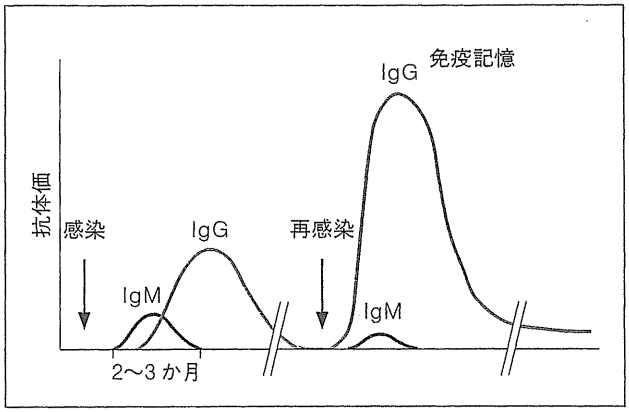


図3　感染によって産生されるIgM抗体価，IgG抗体価の推移

の食細胞と結合する機能をもつ．これらの結合能は後述するオプソニン作用や，補体の活性化能と関係する．Fab部分の抗原結合部位には，無数の抗原に結合できるように変化に富んだアミノ酸配列が存在する．この領域を可変領域(variable region：V領域)といい，H鎖，L鎖にあるV領域をそれぞれVH領域，VL領域と呼ぶ．V領域の多様性は形質細胞へ分化する過程において，VH領域，VL領域で遺伝子の再構成が行われることにより得られ，10^8以上の多様性をもつと考えられている．V領域以外のFab部分とFc部分はほとんど配列がかわらない領域で定常領域(constant region：C領域)と呼ばれる．H鎖には3つC領域(CH1，CH2，CH3)L鎖には1つのC領域(CL)が存在する(図2)．抗体はH鎖のC領域の構造の違いによりIgG，IgA，IgM，IgE，IgDの5つのクラスに分類され，それぞれの抗体は異なった活性をもつ(表2)．

免疫の応答によって最初に産生されるグロブリンは常にIgM抗体であるが，その後，形質細胞内ではC領域の配列が変化してIgG抗体を産生するようになる．この現象をクラススイッチという．IgM抗体は数か月で消失するが，IgG抗体は長く血液中にとどまり，同じ微生物の再感染の予防に作用する．また，再感染が起こったときには刺激を受けた記憶細胞により，大量にIgG抗体が分泌され感染症の予防に働く(図3)．抗体による感染抑制機構は主に以下の3つである．これらを抗体のエフェクター作用と呼ぶ．

(1) 中和活性

抗体がウイルスに結合することで，ウイルスの細胞への結合を抑制したり，あるいはウイルスの細胞内での脱殻を阻止したりすることなどにより，ウイルスの感染性を抑制するような活性をいう．また，毒素などに抗体が結合することにより細胞への侵入を抑制する作用も含まれる．

(2) オプソニン作用

貪食細胞であるマクロファージや樹状細胞は，抗体のFc領域と結合する部位や補体と結合する部位をもっている．Fab領域により病原微生物と結合した抗体が貪食細胞に結合することで，貪食細胞の貪食能を促進させることをオプソニン作

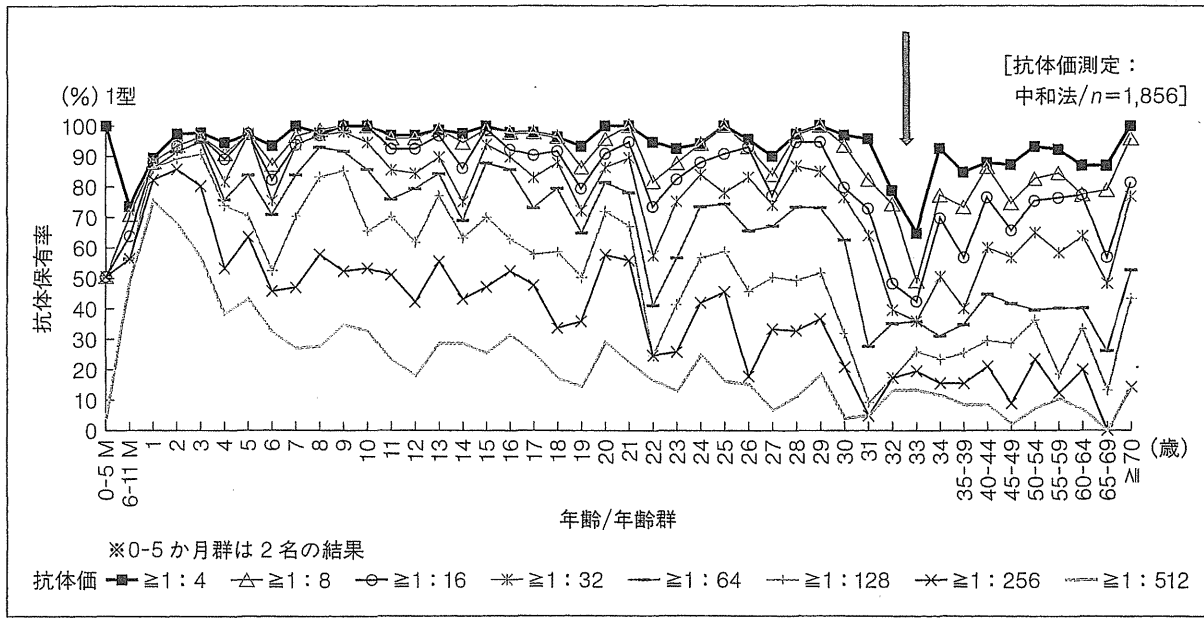


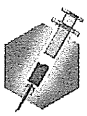
図4　ポリオウイルス1型に対する年齢別抗体分布
矢印の部分が抗体価が低い年齢を示す．

〔文献4）より転載〕

用という．さらに補体が結合することによってオプソニン作用はより強まる．

（3）補体の活性化

ウイルス感染細胞に発現したウイルス抗原に抗体が結合すると補体系が活性化され，ウイルス感染細胞を破壊やオプソニン作用を促進する．



抗体測定の意義

抗体は微生物の感染に対する免疫反応の結果産生され抗原特異的であること，また，その量を表す抗体価は免疫の状態をある程度示すことから，抗体測定は個人や集団の感染症に対する免疫の程度を知るための指標や，病気の原因となる微生物の特定する方法(診断)として用いられている．さらにワクチン開発時の臨床試験における有効性を示す代替指標(サロゲートマーカー)としても用いられることがある．

1．感染症に対する感受性者の調査

感染症，特にワクチンが存在する感染症に対する集団あるいは個人の免疫状態を知ることはワクチンのかかわる感染症対策の基礎となる．厚生労働省が主体となって実施している感染症流行予測調査は，定期予防接種の対象となっている感染症（ポリオ，インフルエンザ，日本脳炎，麻疹，風疹，百日咳，ジフテリア，破傷風)に対する抗体をそれぞれ5,000～6,000人規模で測定し，年齢，地域，性別，ワクチン接種率などの疫学調査結果と併せて検討することで，日本国民のこれらの感染症に対する免疫状況を把握することを目的として行われている．またその調査結果は，将来の流行の可能性を予測し，効果的な対応策を提言するための資料として用いられている．最近では10代において麻疹に対する抗体保有率が低いことが示され，この年代の免疫を強化する目的で2008年から5年間，中学1年，高校3年相当年齢の人に対してワクチンを接種する方針が導入されている．また，1975～1977年に生まれた人においては，原因は不明だがポリオウイルスⅠ型に対する免疫の保有率が低いことが明らかになり，該当する人々にワクチン接種を勧めている(図4)[4]．

一方，個人における感受性検査が必要な例としては，妊娠可能年齢の女性の風疹抗体の測定が挙げられる．妊娠初期の女性が風疹ウイルスに感染すると先天性風疹症候群と呼ばれ，心臓，視力，聴力などに障害をもつ子どもを出産する可能性が高い．したがって，妊娠を予定している女性が風疹抗体価を測定することで風疹に対する免疫状態を確認し，必要に応じてワクチンによって免疫を高めることが勧められている．また医療従事者

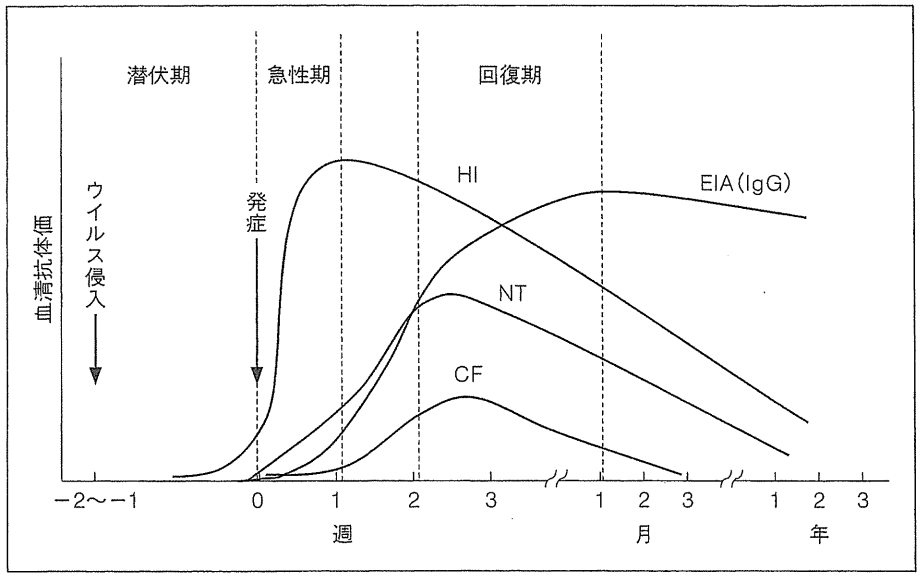


図5　抗体測定法による抗体価の推移の違い

も，麻疹，風疹，おたふく風邪，水痘，B型肝炎などのワクチンで予防できる感染症に対する抗体の保有状況を検査し，必要ならばワクチンを接種することが勧められる．これは自らを感染症から守るだけでなく，治療に集まる患者に感染症を広げないためにも必要な配慮である．学校などの教育機関においても麻疹が流行すれば学級閉鎖，学校閉鎖などの社会的影響の大きい対応が迫られる．最近では入学時に抗体検査，あるいはワクチン接種証明の提出を求める教育機関も増加している．

2．感染症の診断

一般に感染症の診断には，①患者の症状による臨床診断，②疾患の流行性，あるいは罹患者との接触歴などによる疫学的診断，③病原微生物を同定する検査診断，があるが，正確な診断には検査診断を含めた総合的な判断が必要である．検査診断は病原微生物そのもの，あるいはその遺伝子を検出する微生物学的診断と，病原微生物による生体の免疫反応を検査する血清学的診断に分けられる．検体から直接，微生物を検出，同定する微生物学診断は最も重要視すべき診断法であるが，微生物，特にウイルスの分離培養には設備，技術，時間，経費などが必要であり，また分離培養そのものが不可能なウイルスもいることからすべての微生物で実施することはできない．PCRなどによる遺伝子検出法も普及しつつあるが，一般の検査現場で対応できる感染症は限られている．

そこで抗体測定を主とする血清学的診断が用いられている．しかし，血清学的診断は免疫反応により産生される抗体を測定するという間接的な診断方法であり，その特異性，感度には様々な問題があることには留意する必要がある．一般にIgG抗体価によって診断を行う場合，発症後早期(1週間以内)の急性期血清とそれから2週間以上たった回復期血清のペア血清の抗体価を比較し，陽転あるいは回復期において抗体価が4倍以上の上昇が認められたとき，感染があったと診断する．しかしこの方法では回顧的な診断となり，治療開始までに感染源の特定が間に合わない．早急に診断する必要がある場合には，急性期血清を用いて感染初期に産生され，短い期間で消失するIgM抗体価を測定する方法が用いられる．いずれの抗体検査で診断を実施するにしても，抗体の特性を知ったうえで測定結果を慎重に判断する必要がある．

3．ワクチン開発における有効性の検証

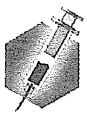
ワクチンは感染症の原因となる微生物に由来する抗原(弱毒した微生物や不活化したウイルス，毒素など)からなり，ヒトや動物にあらかじめ接種し事前に免疫を賦与することで，実際に微生物に曝露されたときにその発症の予防，あるいは症状の軽減を図る製剤である．ワクチン開発における有効性の評価は，ほかの薬剤と同様に臨床試験によって行われる．しかしワクチン以外の薬剤では，主に対象とする病気をもつ患者に投与して，

表3　抗体測定法

	方法・原理	対応できるウイルス	特徴
中和法(NT)	抗体がウイルスを中和することにより感染価が減少することを利用.	分離，培養が可能なウイルス	高感度，特異性が高い，長期間検出，IgG，IgM，IgA を検出.
赤血球凝集抑制試験(HI)	抗体が抗原と結合することにより，抗原の赤血球凝集能の低下することを利用.	HA 活性のあるウイルス	高感度，長期間検出，IgG，IgM，IgA を検出.
補体結合試験(CF)	抗体の存在により抗原抗体複合体が形成され，それに消費される補体の量を溶血反応で測定.	ほとんどすべてのウイルス	感度はあまり高くない，群特異的，非特異反応がある，検出期間は短期，IgG の存在をよく反映する.
受身赤血球凝集反応(PHA)	抗体の存在により抗原を表面に持つ感作血球が凝集することで測定.	ほとんどすべてのウイルス	感度は高い，長期間検出，IgM，IgG を検出，抗体の Avidity の影響を受ける.
受身凝集反応(PA)	抗体の存在により抗原を表面に持つ人工担体が凝集することで測定.	ほとんどすべてのウイルス	PHA より非特異的反応が低い，IgM，IgG を検出，抗体の Avidity の影響を受ける.
酵素免疫法(ELISA)	固相化した抗原に抗体を反応させ，そこに結合する酵素標識二次抗体の量を酵素反応により測定.	ほとんどすべてのウイルス	最も感度が高い，IgG，IgM を別々に測定可能，標準品によりある程度の定量ができる.
蛍光抗体法(IFA)	ウイルス感染細胞に血清を加え，さらに蛍光色素標識二次抗体を加える．蛍光により血清中の抗体を検出する.	分離，培養できるか，感受性動物があるウイルスならば可能	感度は高い，IgG，IgM などを別々に測定可能，実施者の主観が入る場合がある.

治癒，症状の改善などで薬効が検証されるのに対して，ワクチンでは健常人に投与し，目標とする感染症に曝露されるような環境下で実際に発症数が減少するか，あるいは発症しても症状が軽度であるかなどの臨床上の効果を指標にして評価されるべきである.

しかし発生が稀な感染症や発症までに時間がかかる感染症では，実際の臨床症状を指標にしたワクチンの有効性の検証は困難である．また，ワクチン非接種群との比較試験となることから人道的な問題も生ずる．ここにほかの薬剤と異なるワクチンの開発の難しさある．しかし，発症予防効果とワクチンによって誘導される抗体価やそのほかの免疫反応との間の相関性が科学的に確立されているのならば，それらをサロゲートマーカーとして用いてワクチンの効果を推測することができる．最近，日本で承認を受けた麻疹・風疹混合ワクチンや新型インフルエンザワクチンでは，抗体価の上昇を指標に有効性が検証されている.



抗体検査法

日本では多くの抗体測定法が用いられている．それぞれが異なる機構で抗体量を示すので抗体価が同じ意味をもたない．また検体の採取時期によっても抗体価が異なることに留意して，適切な抗体測定法を選択すべきである(図5)．代表的な抗体検査法の概要を以下に記載する(表3).

1．中和法(neutralization test；NT)

抗体がウイルスと結合すると，ウイルスの細胞への感染能や細胞内での増殖能などを減少させることを利用して抗体価を測定する方法である．中和法を実施するためにはウイルスの分離，培養が可能であることが条件となる．ある一定の感染力価をもつウイルスと階段希釈した患者血清を混合後，培養細胞に混合した感染させ，プラーク数などが一定比率以下(通常 50～90%)に減じる最高希釈倍率を用いるプラークリダクション法や，マイクロプレートに培養した細胞で，細胞変成が出現する血清濃度で判定するマイクロタイター法などで中和抗体価を測定する．中和法は一般に感度，特異性が高く，個人の感染防御能と高く相関すると考えられているが，施設，技術，かかる時間などの制約がある．中和法で検出される抗体には IgG，IgM，IgA 抗体がある.

2．赤血球凝集抑制試験(hemagglutination inhibition test；HI test)

ある種のウイルスは動物の赤血球と結合する性

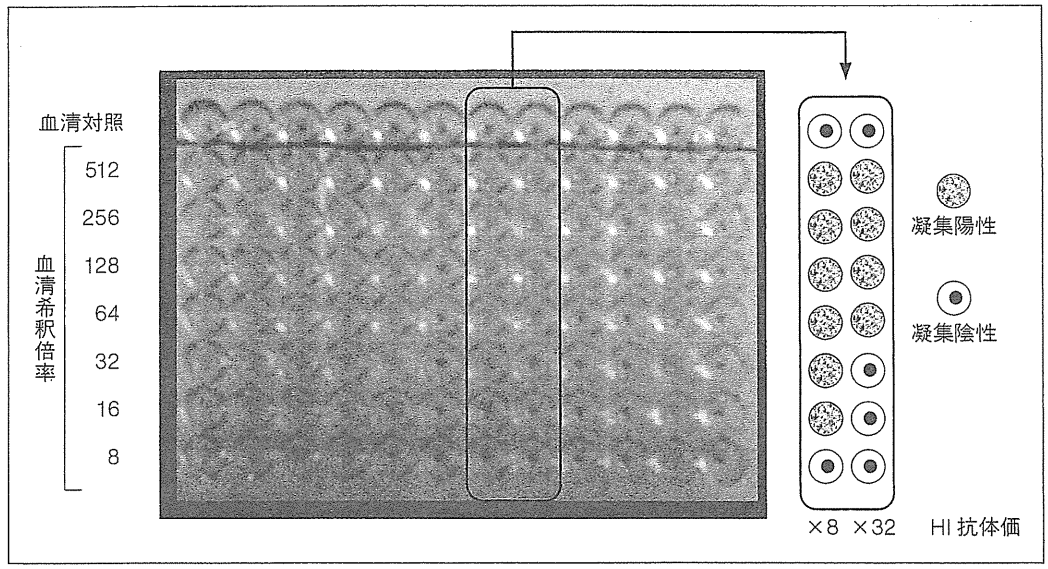


図6 赤血球凝集抑制試験の結果
血清対照：血清中に非特異的なHI活性がないことを確認するため，HA抗原を加えずに反応させる．非特異反応がなければ凝集陰性となる．

質をもつ．これらのウイルス抗原と赤血球を混合するとウイルスを仲立ちにして赤血球が凝集する．この現象を赤血球凝集(Hemagglutination；HA)という．HI試験は抗体がウイルスと結合することでHA能を抑制する性質を利用して抗体価を測定する方法である．血清を2倍階段希釈し，ウイルス抗原と反応させた後，動物の赤血球と反応させる．血清中にウイルス特異的な抗体が存在した場合，抗原と結合してHA活性を抑制する．抗体価はHA活性の抑制を示す最大の血清希釈倍数で表される．HI試験は感度が高く，比較的簡単で一度に多くの検体の測定が可能であるが，HA能をもたないウイルス(HSV，VSV，CMVなど)には使用できない．また，HI抗体価は必ずしも中和抗体価とは一致しない．同種の動物でも血球を採取した個体によって感度が異なることや，血清中に非特異的な血球凝集能がしばしば残ることなどがあり，結果の解釈には注意を要する．感染後，早期に上昇し，長期間持続するため患者の免疫状態の把握や疫学調査に利用されることが多い．HI試験ではIgG，IgM，IgA抗体を検出する(図6)．

3．**補体結合試験**(complement fixation test；CF test)

抗体と抗原が結合して抗原抗体複合体を形成すると，抗体のFc部分に補体が非特異的に結合する性質がある．一方，補体は感作ヒツジ赤血球(溶血素を結合させた赤血球)を溶血させるという性質をもつ．これらの性状を利用して血球の溶血を指標に，検体中に抗原特異的な抗体が存在するかを検査する方法である．事前に56°C，30分間加温し非働化した検体の2倍階段希釈列を作製し，各希釈血清を抗原と混合し，それに補体を加え反応させた後，感作赤血球を添加する．検体中に抗原特異的な抗体が存在する場合，抗原抗体複合体が形成され補体が結合し，反応液中のフリーな補体が減少し溶血が阻止される．抗体価は溶血が阻止される最大希釈血清倍数で表す．感度は中和，HI試験より劣り，特異性も低い．また，中和抗体やHI抗体とは必ずしも相関しない．IgG，IgMは補体結合能をもつので計測できるがIgAはできない．抗原として準備できるほとんどのウイルスで利用できるが，感度，特異性が劣るため感染症の診断にはあまり向かない．

4．**受身凝集反応：受身血球凝集反応**(passive hamagglutination；PHA)，**粒子凝集反応**(particle agglutination；PA)

表面にウイルス抗原を付着させたヒツジ赤血球(PHA)，あるいはラテックス，ゼラチン粒子などの人工担体にウイルス抗原を付着させた感作粒子(PA)が抗体と反応すると赤血球，人工担体が凝集することを利用した抗体検査法である．凝集を起こした最高希釈倍率をもって抗体価とする．簡便で短時間に結果が得られ，感度，特異性に優