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CONTRIBUTION TO LABORATORY DIAGNOSIS OF MUMPS AND PARAINFLUENZA

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Summary. — Specific IgM and IgG antibodies to mumps virus (MV) were detected in sera of mumps-patients by ELISA in agreement with the results obtained by indirect immunofluorescence (IF). Of given sera 37.5% contained IgM reacting in indirect ELISA also with the antigens of parainfluenza virus (PiV) T3. In all patients with respiratory illness over 2 years of age, the significant increase of antibodies to PiV in haemagglutination inhibition (HI) test was in good correlation with serum IgM and IgG antibody levels to PiV T3 determined by ELISA; but, in addition, 30.7% of these sera cross-reacted with MV antigens. The cross-reactions were eliminated by using MV-nucleocapsid antigen in indirect ELISA, or in direct ELISA using the peroxidase-labelled whole virion antigen. In some children under two years of age a discrepancy was observed between the significant increase of serum antibodies in HI and the inability to detect specific IgM antibodies by means of ELISA in their sera. The low-avidity antibodies appearing after primary PiV infection were probably washed off during the ELISA procedure.

Introduction

The reliable detection of IgM and IgG antibodies to mumps virus (MV) is important for evaluation of the efficacy of vaccination programmes as well as for differential diagnosis of atypical MV infections, eg. aseptic meningitis, orchitis or pancreatitis without evident swelling of the parotid gland. The correct interpretation of serological examinations is often complicated by cross-reactions of antibodies to PiV with MV antigens and vice versa. Paired sera of persons with confirmed mumps and/or PiV infections were used to determine the optimal types of antigens and other conditions for ELISA tests to avoid cross-reactions.

Materials and Methods

Antigens. The MV, strain Enders and the PiV T3, strain C 243 — Microbiol. Assoc., Bethesda, Ml. (kindly provided by Dr. Fedová from the Institute of Hygiene and Epidemiology, Praha), were used for preparation of antigens. The MV-whole-virion antigen (PAR) was prepared from

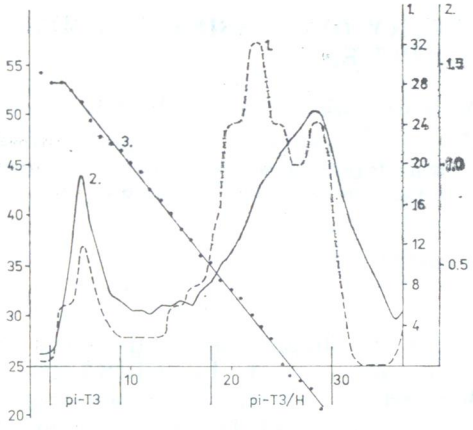

Fig. 1.
Characteristics of PiV-T3 and PiV-T3/H antigens prepared by centrifugation in linear sucrose density gradient
Abscissa: number of fractions; left ordinate: sucrose concentration (%)
1. haemagglutination activity (HA units)
2. optical density at 280 nm
3. sucrose concentration in indicated fraction

allantoic fluids of infected chick embryos by differential centrifugation and ultracentrifugation in linear sucrose density gradient (SDG). The PAR antigen was used in indirect ELISA or, labelled with peroxidase (PAR/px) for direct ELISA. The PAR antigen was also used for further preparation of the MV-nucleocapside antigen (PAR/NC).

The PiV antigens were prepared from infected cultures of monkey kidney cells by sonication, differential centrifugation and ultracentrifugation in linear SDG. As it is evident from Fig. 1, two fractions were used. The first fraction (46—53% sucrose) represented a whole-virion antigen Pi-T3, the second fraction (20—37% sucrose) had high haemagglutinin activity and will be referred to as a crude subunit antigen Pi-T3/H (for details see Grubhoffer *et al.*, 1987).

Sera. Three groups of sera were investigated: 1) Paired sera from 38 persons with respiratory illness caused by PiV infections, confirmed by high levels or significant elevations of antibody titres to PiV in HI. 2) Paired sera of 16 patients with typical mumps, confirmed by detection of high levels of specific serum IgM and IgG by indirect immunofluorescence (IIF). 3) Control group of 14 sera from healthy blood donors.

All sera were inactivated at 56 °C for 30 min and stored in small volumes at —20 °C till use. Rheumatoid factors were removed by immunosorption, as described in a previous report (Fraňková *et al.*, 1985).

The ELISA tests. IgM antibodies were detected by indirect ELISA with the antigens PAR, PAR/NC and Pi-T3/H and by direct ELISA with the antigen PAR/px. IgG antibodies were investigated only by indirect ELISA with the antigens PAR and Pi-T3/H. The optimal dilution of antigens contained 5 μg protein/ml for the PAR antigen and 10 μg protein/ml for all other labelled or unlabelled antigens. Polystyrene flat-bottom microtiter plates (KOH-I-NOOR, Dalečín, Czechoslovakia) were used for the ELISA tests. All components of the reaction were applied in 100 μl amounts per well. After each step of the ELISA procedure the plates were washed 3× with PBS containing 0.1% of Tween 20 (PBS-T). The PBS-T with 1% of bovine serum albumin (BSA) was used for the dilution of peroxidase-labelled conjugates. The sera were diluted in PBS with 1% BSA as follows: 1 : 100, 1 : 500, and 1 : 1000 for the detection of IgM and 1 : 500, 1 : 1000, and 1 : 5000 for IgG. Each dilution of sera was examined in two parallel wells. The examination of control positive and negative serum was included in each plate.

In indirect ELISA the wells were coated with antigens by overnight incubation at 4 °C. The antigen-coated wells were incubated with sera and in the next step with peroxidase-labelled conjugates (SwAHU IgM/Px or SwAHU IgG/Px, Sevac, Praha) always for 2 hr at 37 °C. In the direct ELISA polyclonal capture antibodies (Q-SwAHU IgM, Sevac, Praha) in a concentration of 100 μg/ml were linked by overnight incubation at 4 °C to microtiter plates, pretreated with 2.5% glutaraldehyde (2 hr at room temperature). The plates coated with capture (anti-human IgM) antibodies were incubated with samples of sera, than washed and incubated with labelled antigen PAR/px. The buffers used and the incubation conditions were the same as in the indirect ELISA.

The binding of labelled antibodies or antigens was visualized with 0.03% H_2O_2 and orthophenylendiamine (0.5 mg/ml) in phosphate-citrate buffer (pH 5). After 15 min incubation in the dark at room temperature, the reaction was terminated by addition of 50 µl of 4N H_2SO_4 per well.

Evaluation of ELISA. The optical density (OD) was measured in a spectrophotometer (Microelisa Reader, Dynatech) at a wavelength of 492 nm. The ratio of OD values of specimen serum to control negative serum at the same dilution (S/N) exceeding 2 was considered for positive. To detect IgM by indirect ELISA the critical S/N values were adjusted for particular antigens to the mean values of S/N determined by reaction of sera from blood donors with different antigens (S/N bd). The cutoff level of positivity was determined as S/N bd + 1. The significant increase of antibody level in convalescent serum was defined either by positivity in a higher dilution or by the increase of the S/N values by 1 or more as compared with S/N of acute serum in the same dilution.

Other serological methods: Standard technique was used in the HI tests. The method of IIF was described in our previous report (Fraňková and Sixtová, 1987).

Results

In preliminary box-titrations of PiV antigens higher values of S/N were obtained for IgM detection with Pi-T3/H antigen, than with Pi-T3 antigen, which, on the contrary, was more sensitive for IgG determinations (Fig. 2).

The results of IgM examination were negative in all sera from the control group by direct ELISA. (The S/N ratio never exceeded 2). In indirect ELISA the sera from some blood-donors reacted in all their dilutions with different antigens more intensively, than our negative reference serum. Anyway, all the S/N values, albeit exceeding 2, were substantially lower, than those of any true positive sera (Table 1, Figs 3 and 4). Thus, we had to correct the cutoff values of S/N critical for IgM positivity in indirect ELISA, taking into account the mean values of S/N of control group sera. Under

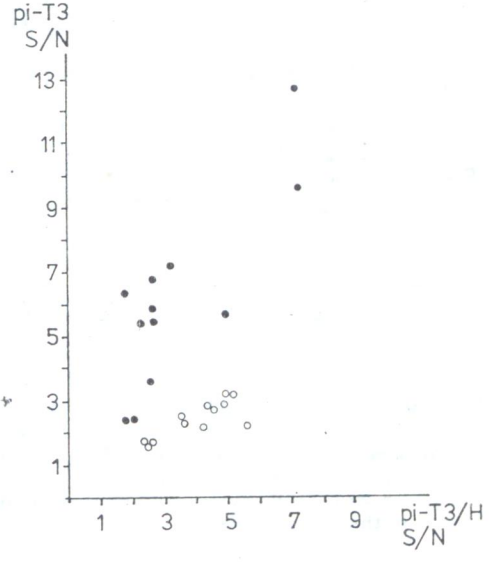

Fig. 2.
Detection of IgM and IgG antibodies directed to PiV-T3 antigen by using ELISA with the PiV-T3 and PiV-T3/H antigens
Abscissa: S/N values of PiV-T3/H antigen; ordinate: S/N values of PiV-T3 antigen
Antibodies used: ○ IgM; ● IgG

Table 1. Results of IgM detection in sera of blood donors: The S/N values obtained in indirect ELISA with different antigens

Antigen	Serum dilution	S/N<2 (number)	Limits of elevated S/N ratios	Mean values of S/N bd	Positive control serum S/N
Par	1 : 100	0	—	—	3.5*
	1 : 500	8	2.2—4.3	3.75	10.2
Par/NC	1 : 100	4	2.3—2.7	2.47	3.5*
	1 : 500	8	2.4—4.4	3.7	8.7
Pi-T3/H	1 : 100	10	2.2—4.3	2.7	3.7*
	1 : 500	7	2.3—3.4	2.8	7.0
	1 : 1000	5	2.4—3.0	2.9	6.6

* OD of the specimen reached the maximum measurable value

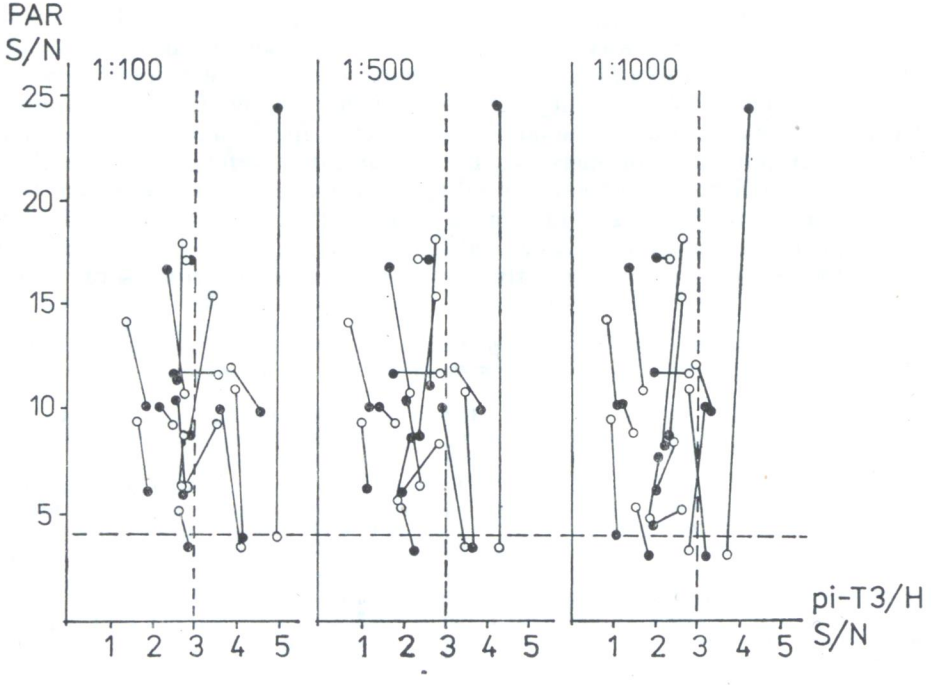

Fig. 3.

IgM antibody response to PAR and PiV-T3/H antigens in patients with mumps
Abscissa: S/N values of PiV-T3/H antigen; ordinate: S/N values of PAR antigen
Dilutions of sera investigated: 1 : 100, 1 : 500, 1 : 1000
Sera: ● acute, ○ convalescent
Serum samples with antibody titre lower than dilution of sera tested with indicated antigen are situated in the region limited by ------

Table 2. Antibody response of patients with mumps as determined by IIF and direct or indirect ELISA with different antigens

Patient no.	Day after onset of disease	IF-IgM	ELISA IgM			IF-IgG	ELISA IgG	
			Par	Par/Px	Pi-T3/H		Par	Pi-T3/H
1	4	+++	1000	1000	–	++++	–	–
	15	+	1000i	1000i	–	++++	5000i	–
2	5	+	1000	1000	1000	++++	5000	1000
	14	++	1000i	1000	1000	++++	5000i	–
3	5	+	1000	1000	1000	+++	5000	500
	12	++	1000	1000	1000	++++	5000i	500
	57	++	100	–	1000	++++	5000	500
4	7	+	1000	1000	–	+++	1000	–
	21	++	1000i	1000i	–	++++	5000i	–
5	6	++	1000	1000	–	+++	–	–
	12	++	1000	1000	–	++++	1000i	–
6	4	+++	1000	1000	–	++++	1000	1000
	33	+	1000	1000	–	++++	5000i	1000
7	32	+++	1000	1000	–	++++	5000	–
	45	+	1000	1000	–	++++	5000i	–
8	7	+	1000	1000	–	++++	nd	500
	12	+	1000	1000i	–	++++	5000	500
9	?	+++	1000	1000	–	++++	5000	1000
	?	++	1000i	1000	–	++++	5000i	1000
10	9	+–	100	500	1000	+–++	–	500
	15	+–++	1000i	1000i	500	++++	5000i	500
	30	++	1000	1000	–	++++	5000	–
11	8	+++	1000	1000	–	++++	5000	–
	29	+++	1000i	1000i	100i	++++	5000i	–
12	4	++	1000	1000	–	+–	500	–
	19	++	1000i	1000i	–	++++	5000i	–
13	11	++	1000	1000	1000	+++	5000	5000
	60	+–++	–	500	500	++++	5000	–
14	11	++	1000	1000	–	++++	5000	–
	19	+++	1000	1000	–	++++	5000i	–
15	3	+–	–	–	–	++	5000	1000
	38	++	1000i	1000i	–	++++	5000i	–
16	7	++	1000	1000	–	+++	1000	–
	44	++	1000	1000i	100i	+++	5000i	500i

Remark to Tables 2–5: Titres given in dilution reciprocals. 100 for IgM, 500 for IgG
Intensity of specific IF: + = weak, but distinct fluorescence, ++++ = bright, very intensive fluorescence
Index i indicates the increase of antibody level as compared with previous serum sample

Table 3. Antibody response determined by HI and ELISA in children under 2 years of age with respiratory disease

Patient no.	Date of collection	HI titre	ELISA — IgM			ELISA — IgG	
			Pi-T3/H	Par	Par/Px	Pi-T3/H	Par
1	28. 2.	64	—	—	—	—	—
	12. 3.	256	—	—	—	—	—
2	18. 6.	256	—	—	—	—	—
	25. 6.	1024	—	—	—	—	—
3	15. 3.	8	—	—	—	—	—
	26. 3.	64	—	—	—	—	—
4	5. 2.	32	—	—	—	—	—
	26. 2.	256	—	—	—	—	—
5	4. 10.	64	—	—	—	5000	—
	18. 10.	512	—	—	—	5000	—
6	12. 8.	32	—	—	—	—	—
	26. 8.	1024	100	—	—	5000i	500i
7	30. 8.	16	500	—	—	—	—
	17. 9.	128	1000i	—	—	—	—

these conditions the results of IgM examinations of sera from blood-donors were all negative with the PAR antigen, and IgM in a titre of 1 : 100 to Pi-T3/H antigen was found in one person.

The antibody response of patients with mumps

In the examination of 34 sera from 16 persons with clinically typical mumps for the presence of IgM and IgG to MV by means of IIF and direct

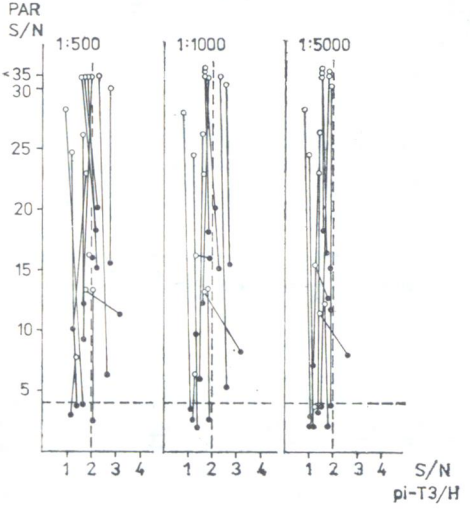

Fig. 4.
IgG antibody response to PAR and PiV-T3/H antigens in patients with mumps
Abscissa: S/N values of PiV-T3/H antigen; ordinate: S/N values of PAR antigen
Dilutions of sera investigated: 1 : 500, 1 : 1000, 1 : 5000
Sera: ● acute, ○ convalescent
Serum samples with antibody titres lower than dilution of sera tested with indicated antigen are situated in the region limited by ------

or indirect ELISA the results were in almost complete agreement. In 2 out of 34 IgM determinations and in 3 out of 33 examinations for IgG the IIF method proved more sensitive.

At the same time 37.5% of sera with IgM to MV reacted as IgM-positive with Pi-T3/H antigen; IgG to this antigen was found in 47% of patients with mumps, but only one increase of IgG level in a convalescent serum sample was observed. The results of the examinations of patients with mumps are summarized in Table 2. The kinetics of antibody responses to MV antigens and cross-reactions with PiV antigens are shown in Figs 3 and 4.

The antibody response of persons with respiratory disease

The results of examinations had to be evaluated separately in different groups of patients.

1) The group of 17 patients with a significant increase of antibodies to PiV-T3 in HI in convalescent sera included samples from 7 children under 2 years of age. Only in two of these children the IgM response to PiV could be demonstrated by means of ELISA, with a rise of specific IgG in only one of convalescent sera. In the rest of the patients of this group serum

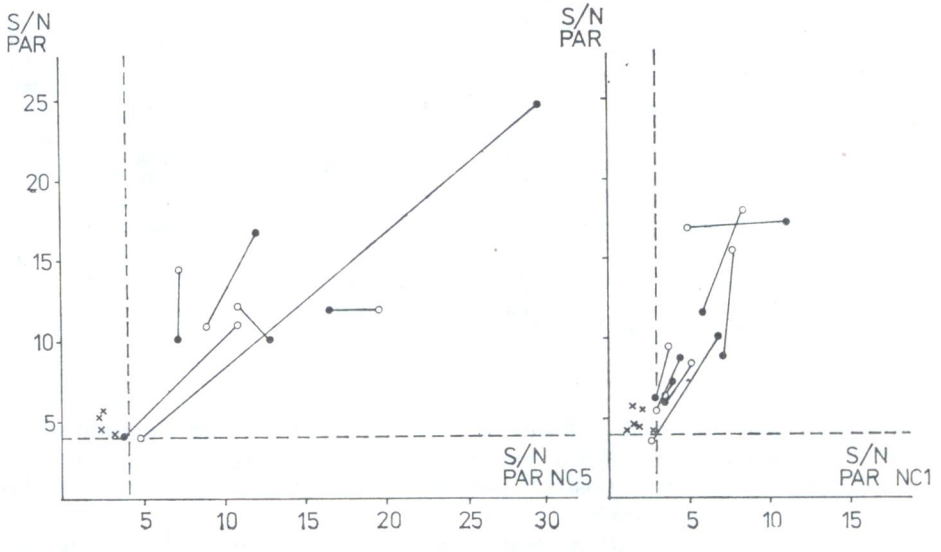

Fig. 5.

Detection of IgM antibodies directed to PAR antigen by using ELISA with PAR and different batches of PAR/NC antigens

Abscissa: I — S/N values of PAR/NC5 antigen, II — S/N values of PAR/NC1 antigen; ordinates: S/N values of PAR antigen

Sera of infected persons: ● acute MV, ○ convalescent MV, × PiV-T3

In the region limited by ------ are situated sera with antibody titre $<1:100$

Table 4. Antibody response determined by ELISA in persons older than 2 years with respiratory disease, with significant increase of antibody titre to different types of PiV in HI

Patient no. serum no.	Significant increase of HI antibody to PiV type	ELISA IgM			ELISA IgG	
		Pi-T3/H	Par	Par/Px	Pi-T3/H	Par
1/1	3	100	—	—	500	—
2		100i	—	—	1000i	—
2/1	3	100	—	—	500	—
2		500i	—	—	500	—
3/1	3	500	—	—	5000	1000
2		100	—	—	5000	1000
4/1	3	500	500	—	5000	1000
2		1000i	1000i	—	5000	1000i
5/1	3	100	—	—	1000	—
2		1000i	1000i	—	5000	1000i
6/1	3	500	—	—	5000	—
2		500i	—	—	5000i	—
7/1	3	—	—	—	1000	—
2		100i	—	—	5000i	500i
8/1	3	—	—	—	1000	—
2		—	—	—	1000	—
9/1	2	1000	1000	—	nd	500
2		1000	1000i	—	nd	500
10/1	2	100	—	—	5000	1000
2		100	—	—	5000	1000
11/1	1	1000	100	—	5000	1000
2		1000	—	—	5000	500
12/1	1,2	1000	100	—	5000	5000
2		1000	100	—	5000	1000
13/1	2	100	—	—	500	—
2		100	—	—	1000i	—
14/1	2	—	—	—	1000	—
2		500i	—	—	500	—
15/1	2	500	—	—	—	—
2		100	—	—	—	—
16/1	1	100	—	—	5000	—
2		100	—	—	500	—

IgM to PiV was detected in 9 out of 10 persons. Significant increase of IgG to PiV in convalescent serum was determined in 5 IgM-positive persons. Out of altogether 11 IgM-positive patients of this group 4 persons were IgM positive with the PAR antigen.

2) The second group of patients included 8 persons with a significant increase of antibody titres to PiV-T1 or T2 in convalescent sera, as determined by HI, with unchanged level of antibody to PiV-T3. All persons from this group were IgM-positive to Pi-T3/H by ELISA, 3 of them cross-reacting as IgM-positive with the PAR antigen.

3) Additional 12 patients showed antibody titres to PiV T3 equal to-, or exceedig HI titre of 512 in both serum samples. The aethiological role of PiV T3 was verified in 7 patients from this group by detection of IgM to

Pi-T3/H. Out of 14 paired sera of the IgM-positive patients five cross-reacted as IgM-positive with the PAR antigen as well.

The IgM antibodies to PiV were found althogether in 49 samples out of 74 sera of persons with respiratory disease by indirect ELISA. Of these samples containing IgM class antibody to Pi-T3/H, were IgM-positive with PAR antigen (30.6%). No cross-reactions were observed in direct ELISA with PAR/px, or in indirect ELISA with PAR/NC antigens (Fig. 5, Tables 3, 4 and 5).

Discussion

We paid special attention to specific and cross-reacting antibodies of the IgM class, as their reliable determination may play a decisive role in diagnosis and differential diagnosis of MV or PiV infections. The rheumatoid factors were removed from all sera before examination to reduce the occurrence of unspecific findings. Nevertheless, in agreement with Ukkonen *et al.* (1980), Tuokko (1984) and others, if the value of S/N limiting positivity was 2, the sera of some healthy persons reacted in our indirect ELISA tests as IgM-positive. Such false-positive findings of IgM are explained by the reaction of sera of some individuals with nonspecific components of antigens (Kuno-Sakai *et al.*, 1984, Tuokko, 1984). The S/N values in false--positive IgM findings were negligible as compared with S/N of true positive sera, so when the modified limiting S/N values were used as a criterion of positivity, specific results were obtained.

With modified cutoff-levels of the S/N ratio, determining true positivity of IgM findings, all examinations of IgM, except one, were negative by indirect ELISA in healthy persons, which is in agreement with the results of direct ELISA. Under equal conditions, the determinations of specific IgM even in the week-reacting persons with proven infection were based on S/N values substantially exceeding our modified cutoff S/N positivity limits. We suppose that the determination of IgM in our investigations was specific and sensitive enough and that the cross-reactions observed in indirect ELISA tests were due to common antigens between MV and PiV.

The antibodies cross-reacting in indirect ELISA were predominantly IgM. In contrast to this, Meurman *et al.* (1982), using whole-virion antigens found cross-reacting antibodies to MV and PiV mainly in the IgG class. This discrepancy can be explained by different types of antigens used. As has been shown in our preliminary titrations, the Pi-T3/H antigen, containing largely the viral surface glycoproteins reacted preferentially with IgM antibodies, while specific IgG was bound mostly by the whole-virion antigen. These observations illustrate the dependence of results on the type of antigen used in indirect ELISA tests.

Örvell *et al.* (1986) used monoclonal antibodies to several viral polypeptides in searching for common antigen determinants between MV and PiV without success. Nevertheless, in human natural infections the cross-reacting antibodies can be often found, complicating the evaluation of different serological tests (Lennette *et al.*, 1963, van der Logt *et al.*, 1982, Julkunen,

Table 5. The antibody response determined by ELISA in persons with respiratory disease and with a significant increase of HI antibody to PiV T3 in convalescent sera

Patient no.	Date of serum collect.	HI titre	ELISA IgM			ELISA IgG	
			Pi-T3/H	Par	Par/Px	Pi-T3/H	Par
1	December						
	2. 12.	4096	100	n.d.	—	nd	5000
	11. 12.	4096	500i	100i	—	nd	5000
2	November, December						
	13. 11.	2048	100	100	—	—	500
	4. 12.	1024	100	—	—	1000	—
3	November, December						
	13. 11.	4096	—	—	—	—	1000
	4. 12.	4096	—	—	—	—	1000
4	December						
	2. 12.	4096	1000	100	—	5000	1000
5	October ?	4096	100	—	—	500	500
	18. 10.	256	100	—	—	1000	—
	?	512	500i	—	—	1000	—
6	November, December						
	26. 11.	512	—	—	—	1000	—
	9. 12.	512	—	—	—	1000	—
7	November						
	12. 11.	4096	100	—	—	1000	—
	22. 11.	4096	100	—	—	500	—
8	November						
	4. 11.	4096	—	—	—	1000	—
	14. 11.	2048	—	—	—	5000i	—
9	November, December						
	27. 11.	512	—	—	—	—	1000
	11. 12.	512	—	—	—	—	1000
10	November						
	12. 11.	2048	—	—	—	5000	—
	21. 11.	1024	—	—	—	5000	—
11	?	1024	100	—	—	1000	1000
	?	2048	100	—	—	5000i	1000
12	November						
	13. 11.	4096	500	500	—	1000	1000
	?	4096	1000i	1000i	—	5000	1000

1984). These cross-reactions may arise from only partial identity or similar configuration of some epitopes of MV and PiV antigens, which may gain importance in the enhanced anamnestic antibody response after repeated infections with PiV. This presumption is supported by the irregularity of the occurence of these cross-reactions. In our investigations cross-reactivity was observed only in about 1/3 of patients with mumps or PiV infections and all of them were more than 2 years old.

Our observations are in agreement with the results of Julkunen (1984), who found by indirect ELISA in 5 out of 10 patients with proven mumps a significant rise of antibodies to surface glycoproteins of PiV-T3. Using the radioprecipitation with rabbbit immune sera the author showed that

the target of cross-reacting antibodies between MV and PiV are mainly viral glycoproteins. This was the reason for the use of the PAR/NC antigen in our reexamination of persons with PiV infection exhibiting IgM positivity to PAR antigen. As expected, the cross-reactions were eliminated by the use of the PAR/NC antigen.

The finding of nonspecific and cross-reacting IgM antibodies was eliminated in direct ELISA, using captured antibodies and the peroxidase-labelled whole-virion antigen. Similar experience with high specificity of the direct ELISA system was described by many others (e.g. Schmitz, 1982 or Gut *et al.*, 1985). The elimination of unspecific reactions in the direct ELISA system may be explained by higher weight and size of labelled whole-virion antigen as compared to immunoglobulin molecules. We suppose that after application of labelled antigen on the antibody layer, only the firm, specific binding can withstand the washing procedure that follows in direct ELISA.

A similar mechanism may be involved in occasional discrepancies observed between the results of HI or complement fixation and indirect ELISA (Ukkonen *et al.*, 1980). In our investigations such a discrepancy was found in some children under 2 years of age. We suppose, that in newborns and very small children the immature immune system may respond to primoinfection with low-avidity antibodies. These can be easily washed away in ELISA procedure, but may be fully operative in neutralization of haemagglutinins or fixation of complement.

In accordance with van der Logt *et al.* (1985) and others, the cross-reactions in HI as well as in IgM determination by ELISA were found in our investigation between different types of PiV. Most patients with a significant increase of antibodies to PiV-T1 or T2 in HI had high levels of antibodies to PiV-T3 in both acute and convalescent sera while IgM antibodies to PiV-T3 were demonstrated in all sera. The titres of antibodies to PiV-T3 in HI often exceeded the titres to the type of PiV confirmed as aethiological agent by seroconversion. This can be explained by an anamnestic response to the most often occuring T3 in the course of primary infections with an other type of PiV. From this point of view, our results illustrate understanding the dependence of the host's immunological response on his previous experience with infections caused by microorganisms bearing related or similar antigenic epitopes.

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