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Characterization of genotype-specific epitopes of the HN protein of mumps virus

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The SBL-1 strain of mumps virus, grouping with genotype A on the basis of the small hydrophobic protein gene sequence, was grown in the presence of three different monoclonal antibodies. The monoclonal antibodies were directed against the haemagglutinin–neuraminidase (HN) protein and they inhibited haemagglutinating activity and infectivity of the virus. The HN genes of the nine neutralization-escape virus mutants were sequenced and the predicted amino acid sequences were compared with that of the parental virus. Amino acid substitutions were found at positions 269, 352 and 354, respectively, of the 582 amino acid long protein. The three monoclonal antibodies did not react with 35 virus strains isolated in Stockholm during the years 1970 to 1985. Thirteen and four of the strains were found to belong to the D and C genotypes, respectively. A type-specific neutralization antibody response was also found in sera of rabbits hyperimmunized with purified virions of genotype A and D. The genotype-specific difference in neutralizing activity in mice and rabbits was not corroborated by an overall difference in the amino acid sequence of the HN protein of the different genotypes. Further studies are needed to explore the efficacy of mumps virus vaccines for protection against homologous and heterologous genotypes of mumps virus.

Introduction

Mumps virus belongs to the family *Paramyxoviridae*, genus *Rubulavirus* (Rima *et al.*, 1995). The single-stranded mumps virus genomic RNA contains seven genes in the following order on the genome map; the nucleocapsid (N), phospho (P), membrane (M), fusion (F), small hydrophobic (SH), haemagglutinin–neuraminidase (HN) and large (L) protein genes (Elango *et al.*, 1988; Elliott *et al.*, 1989). The SH gene of mumps virus encodes a presumed viral protein of 57 amino acids in length. Comparisons of the nucleotide sequences of the SH gene from different mumps virus isolates around the world have shown the existence of five genotypes which have been named A to E (Yeo *et al.*, 1993; Künkel *et al.*, 1994, 1995; Örvell *et al.*, 1997). The existence of a sixth genotype has been reported by Ströhle *et al.* (1996). Recently isolated virus strains in Europe have been found to belong to the A, C and E genotypes (Yeo *et al.*, 1993, Künkel *et al.*, 1994, 1995; Örvell *et al.*, 1997). In Japan, only genotype B has been found and this genotype has not yet been found in Europe or the USA among naturally circulating stains (Takeuchi *et al.*, 1991; Yeo *et al.*, 1993; Örvell *et al.*, 1997).

Strain variations in the nucleotide sequence have also been described for the P protein (Yamada *et al.*, 1989), F protein (Takeuchi *et al.*, 1989; Forsey *et al.*, 1990) and HN protein genes (Waxham *et al.*, 1988; Kövamees *et al.*, 1990; Yates *et al.*, 1996). Antigenic diversity identified by monoclonal antibodies has been reported for the P, N, F and HN proteins (Örvell, 1984; Rydbeck *et al.*, 1986; Yates *et al.*, 1996). Some monoclonal antibodies with high neutralizing activity directed against the HN protein of the SBL-1 strain (genotype A) and the Urabe strain (genotype B) have been found to react with the homologous but not heterologous virus strains (Örvell, 1984; Yates *et al.*, 1996).

The aim of the present study was to investigate B cell epitopes on the SBL-1 HN protein that were capable of inducing neutralizing antibodies and also to study the structure of corresponding epitopes in heterologous genotypes.

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Methods

■ **Viruses.** The SBL-1 strain of mumps virus isolated in Stockholm in 1971 and 35 virus stains isolated in the laboratory from 1970 to 1985 were used in the study (Örvell *et al.*, 1997). The virus strains were grown

0001-4993

in Vero cells maintained in Eagle's minimal essential medium (MEM) containing 2% foetal calf serum.

■ **Antibodies.** Eight monoclonal antibodies directed against the HN protein of the SBL-1 strain of mumps virus were used. The antibodies have been characterized by serological analysis and competitive ELISA in a previous study (Örvell, 1984). One group of four antibodies (group III), numbers 2041, 5500, 2072 and 2075, could inhibit haemagglutinating activity and they also exhibited the highest neutralizing titres compared to other antibodies. Other antibodies (group IV), numbers 2034 and 2082, inhibited neuraminidase activity, but did not block haemagglutinating activity. Five rabbits were hyperimmunized with purified virions of the SBL-1 strain, Kilham (genotype A) and the RW (genotype D) strain of mumps virus. The procedures for purification of virions and hyperimmunization of rabbits have been described previously (Örvell & Norrby, 1980).

■ **Production and characterization of neutralization escape virus mutants.** Three times plaque-purified virus preparations of the SBL-1 strain in concentrations of 10^5 to 10^6 $TCID_{50}$ per 0·1 ml were mixed with equal volumes of undiluted ascites material in a tissue culture tube. The tube was shaken and the mixture was incubated at room temperature for 1 h. After that time period 0·1 ml was inoculated on a monolayer of Vero cells in 35 mm Petri dishes. The plates were incubated at 37 °C for 1 h and then a 3 ml overlay of 0·5% agar in HEPES lactalbumin hydrolysate containing 1% (v/v) of the same undiluted monoclonal antibody was added. After 7 days incubation in a CO_2 chamber the Petri dishes were inspected for plaques. The number of plaques formed in the presence of monoclonal antibodies was reduced in the order of 10^{-5} in comparison to the number of plaques in the control preparations grown in the absence of monoclonal antibodies. Plaques that had formed in the presence of monoclonal antibodies were picked up with a Pasteur pipette, grown on Vero cells and virions were purified for use in ELISA experiments.

■ **Immunofluorescence (IF) analysis.** The procedure for IF analysis was similar to that described previously (Rydbeck *et al.*, 1986). Vero cells infected with the different strains of mumps virus were transferred to object glasses and the cells were air dried. The object glasses were fixed in cold (— 20 °C) acetone. The monoclonal antibodies were used at 1:10 and 1:50 dilutions of the original ascites fluid.

After incubation with the antibodies at 37 °C for 30 min., the glasses were washed with PBS after which goat anti-mouse fluorescein-labelled antibodies were added and the incubation was repeated. After washing, Evans' blue at a final concentration of 0·03% was added and the preparations were examined with a fluorescence microscope.

■ **ELISA.** The technique for ELISA has been described previously (Örvell, 1984). Purified virions of the SBL-1 strain and the nine virus mutants were used to coat wells of plastic plates. Two μg of viral protein in 100 μl of buffer was used for coating each well. The titre obtained with the different monoclonal antibodies was expressed as the highest 10-fold dilution that gave an absorbance (*A*) value of more than 0·2 (Örvell & Grandien, 1982).

■ **Neutralization assays.** The procedure for end-point neutralization has been described previously (Örvell, 1976). Serial twofold dilutions of rabbit hyperimmune sera in a volume of 0·15 ml were mixed with an equal volume of virus containing 100 to 200 $TCID_{50}$/0·1 ml. The mixtures were shaken and incubated at 37 °C for 1 h. After that time period 0·1 ml of the antigen–antibody mixtures was inoculated on Vero cells in tissue culture tubes; two tubes were inoculated per antibody dilution. The inoculated tubes were incubated at 37 °C. The tubes were inspected for cytopathic effect and final readings were made after 7 days incubation.

■ **Nucleotide sequencing.** Selected areas of the HN gene of the nine virus mutants were sequenced. Isolation of mumps virus RNA and PCR amplification was performed essentially as has been described for hepatitis C virus by Yun *et al.* (1993). Primers for nested PCR of selected fragments of the HN gene of the SBL-1 strain of mumps virus (Kövamees *et al.*, 1989) were chosen using the Oligo version 5.0 program (NBI, Plymouth, MN, USA). For each fragment one of the inner primers was 5′-biotinylated and the other was coupled at the 5′ end with a universal M13 primer sequence (CGACGTTGTAAAACGGCCAGT). Direct DNA sequencing was performed from the biotinylated PCR product which was immobilized onto streptavidin-coated magnetic beads (Dynal). Single-stranded DNA was generated by denaturation with 0·1 M NaOH. The sequencing was carried out using Cy5-labelled M13 universal primer and the Autoread (Pharmacia) kit on an automated laser fluorescent sequencer (ALF Express, Pharmacia).

Results

Antigenic comparison of the HN protein of different genotypes

Eight neutralizing monoclonal antibodies directed against the HN protein of the SBL-1 strain of mumps virus (Örvell, 1984) were used for comparison of B cell epitopes between genotypes A, C and D (Table 1). The antibodies had previously been characterized for their ability to block different biological activities of the virus. Group II blocked haemolytic activity but not haemagglutinating or neuraminidase activity, group III blocked haemagglutinating and haemolytic activity and group IV could inhibit neuraminidase and haemolytic activity but not haemagglutinating activity. The antibodies belonging to group III exhibited higher neutralization titres than antibodies belonging to the other two groups.

The four antibodies belonging to group II and IV reacted with all 35 mumps virus strains isolated in Stockholm from 1970 to 1985. Seventeen of the isolates had been genotyped and shown to belong to the C and D genotypes (Table 1, Örvell *et al.*, 1997). In contrast, three of four monoclonal antibodies belonging to group III did not react with the 35 mumps virus isolates. Monoclonal antibody 2075 reacted with all 35 mumps virus strains isolated in Stockholm from 1970 to 1985.

Characterization of virus escape mutants

The three monoclonal antibodies which reacted with the SBL-1 strain, but not with the virus isolates belonging to the C or D genotypes, were used for production of virus escape mutants. Nine virus escape mutants were produced. There was no observed difference in the growth capacity or cytopathic effect in comparison of the nine virus mutants and the parental virus. Virus mutants 310, 311 and 312 were obtained with antibody 2041, 320 and 321 were obtained with antibody 5500 and virus mutants 323, 324, 326 and 327 were obtained

Table 1. Immunofluorescence reactivity of different monoclonal antibodies in tests with 18 strains of mumps virus

Isolate	Year of isolation	Genotype	Immunofluorescence with monoclonal antibody no. 1992 (II*) 5374 (II)	2041 (III) 5500 (III) 2072 (III)	2075 (III)	2034 (IV) 2082 (IV)
SBL	1971	A	+	+	+	+
V1	1970	D	+	−	+	+
V3	1970	D	+	−	+	+
F26068	1970	D	+	−	+	+
V4	1971	D	+	−	+	+
V5	1971	D	+	−	+	+
V6	1971	D	+	−	+	+
V8	1971	D	+	−	+	+
V10	1972	D	+	−	+	+
V11	1973	D	+	−	+	+
F50647	1978	D	+	−	+	+
V27	1983	C	+	−	+	+
V28	1983	D	+	−	+	+
V29	1983	C	+	−	+	+
V31	1984	C	+	−	+	+
V33	1984	D	+	−	+	+
V34	1984	C	+	−	+	+
V35	1985	D	+	−	+	+

* The antibodies were grouped according to their ability to block different biological activities of mumps virus (Örvell, 1984).

Table 2. Reactivity measured by ELISA of different monoclonal antibodies in tests with nine virus escape mutants

Monoclonal antibody no.	Epitope*	ELISA reactivity with virus mutant no. 310	311	312	320	321	323	324	326	327
1992 (II)	1	$+(10^4)$	+	+	$+(10^4)$	+	$+(10^3)$	+	+	+
5374 (II)	2	$+(10^4)$†	+	+	$+(10^4)$	+	$+(10^4)$	+	+	+
2041 (III)	3	$-(< 10^2)$	−	−	$-(< 10^2)$	−	$+(10^5)$	+	+	+
5500 (III)	3	$+(10^5)$	+	+	$-(< 10^2)$	−	$+(10^5)$	+	+	+
2072 (III)	4	$+(10^5)$	+	+	$+(10^5)$	+	$-(< 10^2)$	−	−	−
2075 (III)	5	$+(10^5)$	+	+	$+(10^5)$	+	$+(10^5)$	+	+	+
2034 (IV)	6	$+(10^5)$	+	+	$+(10^5)$	+	$+(10^5)$	+	+	+
2082 (IV)	6	$+(10^5)$	+	+	$+(10^5)$	+	$+(10^5)$	+	+	+

* The epitopes were previously characterized by competitive ELISA (Örvell, 1984).
† End-point titre in ELISA.

with antibody 2072 (Table 2). The virus mutants did not react with the antibody that was used for selection of the mutant. Monoclonal antibody 5500 reacted with virus mutants 310, 311 and 312. This result implied that the two antibodies 2041 and 5500, which were previously believed to react with the same epitope by competitive ELISA (Örvell, 1984), were in fact not identical antibodies. In previous work three virus escape mutants of the Kilham strain were produced with antibody 5500 and a presumed epitope was located around amino acid 352 to 360 (Kövamees *et al.*, 1990). The nine virus mutants and

Strain	Genotype	Sequence
SBL	A	345-NPYNPCSGPP QELDQRALRS YFPRYFSSRR VQSAFLVCAW NQILV-389
310	A	.....T ....
311	A	.....T ....
312	A	.....T ....
320	A	.....E.. ....
321	A	.....E.. ....
323	A	....
324	A	....
326	A	....
327	A	....
V1	D	.....Q .D..... ..S.L.N... ....
V3	D	.....Q .D..... ..S.L.N... ....
V4	D	.....Q .D..... ..S.L.N... ....
V5	D	.....Q .D..... ..S.L.N... ....
V28	D	.....Q .D..... ..S.L.N... ....
V35	D	.....Q .D..... ..S.L.N... ....
V27	C	.....Q .D..... ....
V29	C	.....Q .D..... ....
V31	C	.....Q .D..... ....

Fig. 1. Deduced amino acid sequence of the HN protein from amino acid positions 345 to 389 in nine virus escape mutants and nine genotyped virus isolates. Nucleotide sequencing was performed after a nested PCR reaction. The inner primers were 5′ TTGCCCAATAGTACACTAGGA 3′ mRNA sense, nucleotide positions 1056 to 1076 (SBL-1 HN gene, GenBank accession no. M55065) and antisense 5′ GCTCGCAATTTGTAACTAGG 3′, positions 1238 to 1257.

Strain	Genotype	Sequence
SBL	A	240-NRKSCSIATV PDGCAMYCYV STQLEANDYA GSSPPTQKLT LLFYNDTIT ERTISPS-295
310	A	....
311	A	....
312	A	....
320	A	....
321	A	....
323	A	....T ....
324	A	....T ....
326	A	....T ....
327	A	....T ....
V1	D	....TD... ....V. .....
V3	D	....TD... ....V. .....
V4	D	....TD... ....V. .....
V5	D	....TD... ....V. .....
V28	D	....TD... ....V. .....
V35	D	....TE... ....V. .....
V27	C	....TE... ....V. .....
V29	C	....TD... ....

Fig. 2. Deduced amino acid sequence of the HN protein from amino acid positions 240 to 295 in nine virus escape mutants and eight genotyped virus isolates. Nucleotide sequencing was performed after a nested PCR reaction. The inner primers were 5′ CCAATATCTCAGTGATGGCCTGAAT 3′ mRNA sense, nucleotide positions 773 to 797 (SBL-1 HN gene, GenBank accession no. M55065) and antisense 5′ CCCACTCCTGGCACCAAAGT 3′, positions 984 to 1003.

nine virus isolates that did not react with the three monoclonal antibodies were sequenced and the amino acid sequence was deduced around the region of the presumed epitope (Fig. 1). Virus mutants 320 and 321 both contained a mutation at amino acid position 352, where a glycine was substituted for a glutamic acid. Virus mutants 310, 311 and 312 contained a mutation at position 354, where a proline was substituted for a threonine. Virus mutants 323, 324, 326 and 327 did not exhibit any mutation in this region. All group C and D isolates had different amino acids at positions 354 and 356, glutamine instead of proline at position 354 and aspartic acid instead of glutamic acid at position 356. Taken together, these results could explain why the two antibodies 2041 and 5500 could not bind to virions of genotype C or D. In the virus strains belonging to the D genotype the same three amino acids, serine, leucine and asparagine, were found at amino acid positions 367, 369 and 371, respectively, and these amino acids were not found in genotype C.

In a previous study it was shown that the antibody 2072 did not bind to the Enders or the Kilham strain of mumps virus, two other members of the A genotype (Örvell, 1984). Comparison of the amino acid sequence of the SBL-1, Enders

Table 3. End-point neutralizaiton titres in rabbit hyperimmune sera directed against the A or D genotype of mumps virus

Rabbit hyperimmune serum no.	Immunized with strain:	Genotype	Neutralization titre with: SBL(A)	V6(D)	V27(C)
201	SBL	A	640	320	160
202	SBL	A	640	320	160
203	Kilham	A	640	320	320
204	RW	D	80	1280	320
205	RW	D	80	1280	640

and Kilham strain and known C and D genotypes of mumps virus revealed that SBL-1 contained two unique amino acids at position 265 and 266 (Waxham *et al*., 1988; Kövamees *et al*., 1989; Elliott *et al*., 1989; Yates *et al*., 1996). The nine virus mutants and eight of the virus isolates were sequenced in this area of the gene and the amino acid sequence of the protein was deduced (Fig. 2). The four virus mutants 323, 324, 326 and 327 produced with monoclonal antibody 2072 all exhibited an amino acid substitution at position 269, an alanine was exchanged for a threonine. As expected the other five virus mutants did not show any mutation in this region. The eight virus isolates that were examined had a threonine at position 265 and an aspartic acid or a glutamic acid at position 266 which were different from the SBL-1 strain.

Analysis of neutralizing antibodies in rabbit hyperimmune sera

The findings described above suggested that the monoclonal antibodies blocking haemagglutination were type-specific and were directed against areas of the protein where amino acid variation occurred. It was considered of interest to investigate if polyclonal sera contained type-specific neutralizing antibodies. A type-specific antibody response occurred when sera from rabbits hyperimmunized with genotype A and D of mumps virus were tested (Table 3). A pronounced difference was found when genotypes A and D were compared. Type-specific neutralization antibodies dominated over cross-reactive antibodies. The neutralization titres in tests with genotype D and C showed less pronounced difference.

Discussion

In the present study it was shown that mumps virus exhibited a type-specific neutralization antibody response. Three out of four monoclonal antibodies, blocking haemagglutination of the SBL-1 strain of mumps virus, did not react with the C or D genotypes. The three monoclonal antibodies selected virus mutants with amino acid changes at amino acid positions 269, 352 and 354, respectively. The lack of binding of these antibodies to the C and D genotypes could be explained by the finding of the existence of different amino acids at positions 265, 266, 354 and 356 compared to the SBL-1 stain. In contrast to these findings it was found that monoclonal antibodies blocking neuraminidase activity, but not haemagglutinating activity, reacted with both genotypes C and D. Similar findings were made in the study by Yates *et al*. (1996) with monoclonal antibodies directed against the HN protein of the Urabe strain (genotype B).

The results from studies on mouse monoclonal antibodies correlated with results obtained with polyclonal sera. In rabbit hyperimmune sera both type specific and broadly reactive neutralization antibodies were found. Type-specific antibodies dominated over cross-neutralizing antibodies. The 582 amino acid long sequence of the HN protein of the SBL-1 strain of mumps virus differs in 30 and 25 amino acid positions compared to the RW and two genotype C strains, respectively (Yates *et al*., 1996).

It is therefore probable that the quantitative and/or qualitative neutralization antibody response is directed against epitopes where structural differences occur between genotypes. Also, in a recent study on respiratory syncytial (RS) virus, another member of the paramyxovirus family, it was shown that although the overall amino acid sequence did not show pronounced differences, amino acid differences that exist in the G envelope protein of different strains of bovine RS virus have a profound effect upon antibody recognition (Furze *et al*., 1997). Two such epitopes were identified in the present study on mumps virus. When different members of the C and D genotypes, isolated over a 15 year period, were compared, it was shown that that the amino acid sequences in the regions of the two epitopes were highly conserved in both genotypes. In previous studies with virus escape mutants produced in the presence of neutralizing monoclonal antibodies multiple amino acid alterations have been found although only one antibody was used for selection of virus mutants (Kövamees *et al*., 1990; Yates *et al*., 1996). This phenomenon was not observed in the present investigation. The different mutants that were produced with the same monoclonal antibody showed the same amino acid substitution and only one amino acid was changed in the epitope. In a previous study with monoclonal antibody 5500 three virus escape mutants were produced (Kövamees *et al*., 1990).

Amino acid changes were found at positions 239, 297, 329, 352, 353, 358, 360 and 523 with unique substitutions for each of the three virus mutants. In this study the two virus mutants produced with antibody 5500 showed an amino acid change at position 352, but no amino acid changes at positions 239, 297, 353, 358 and 360 were found. Possible amino acid mutations at positions 329 and 523 were not examined in the present study, but the occurrence of mutations at these two points would not be expected to be of biological importance, due to the presence

of amino acid homologies that exist in these regions in different genotypes. Moreover, the amino acid mutations at positions 329 and 523 were only found in one and two, respectively, of the three virus mutants examined in the work by Kövamees *et al*. (1990). In the study by Yates *et al*. (1996), one virus mutant produced with one monoclonal antibody exhibited an amino acid substitution at position 359 which most probably relates to the epitope at amino acid positions 352 and 354 described in the present study.

A type-specific neutralization response similar to that described in the present study was also reported by Yates *et al*. (1996) in studies of human sera after vaccination with mumps virus and in sera obtained from unvaccinated blood donors aged between 18 and 65 years. When comparing the results from the two studies it appears that the type-specific response was more pronounced in monospecific rabbit hyperimmune sera than in human sera. The difference observed is likely to be due to the fact that the human sera are post-infection while rabbit sera are not. However, at this point it cannot be ruled out completely that the human subjects may have undergone subclinical reinfections with different genotypes of mumps virus causing a broadening of the immune response. Previous studies have shown that more than one genotype can circulate at the same time in the same geographical location, and also that the circulation of different genotypes in one geographical location can change with time (Künkel *et al*., 1995; Ströhle *et al*., 1996; Örvell *et al*., 1997; Afzal *et al*., 1997).

The findings in the present study raise the question if a vaccine containing one genotype of mumps virus is optimal for protection against the other genotypes of mumps virus. In Sweden the Jeryl Lynn (genotype A) strain has been used for vaccination for more than 25 years. This vaccine may be insufficient for effective protection against the C and D genotypes of mumps virus. Further studies will be necessary to clarify this issue.

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