Antibodies specific for measles virus envelope antigens and autoantibodies in patients with chronic active hepatitis

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SUMMARY Distinctly increased levels of antibodies to measles virus envelope antigens haemolysin and haemagglutinin were found in the sera of patients with chronic active hepatitis compared with a normal control group, using immunofluorescence and functional tests. Similarly, a higher incidence of smooth muscle antibody of both IgG and IgM classes was observed in the patients and an important correlation was found between haemolysin antibodies specific for measles virus and smooth muscle antibody of IgG and IgM classes. In contrast, there was no such correlation between the virus specific haemolysin antibodies and antinuclear antibodies. The increased levels of antibodies to measles virus envelope antigens and of autoantibodies may reflect defects in immunoregulation rather than persistent infection with measles virus.

Increased antibody titres to measles virus have often been observed in the sera of patients with chronic active hepatitis who are negative for hepatitis B surface antigen (HBs Ag).¹⁻⁷ Similarly, a higher incidence of autoantibodies, especially smooth muscle antibodies and antinuclear antibodies, has also been detected in the sera of these patients.⁸⁻¹⁰

Measles virus, a member of the genus *morbillivirus* of the family *Paramyxoviridae*, both haemagglutinates and haemolyses rhesus monkey erythrocytes but lacks neuraminidase.¹¹ Biological and biochemical analyses of measles virus have shown that the two antigens located on the surface of the virus envelope are haemagglutinin and haemolysin, while the major antigen, nucleocapsid, is held internally within the virion. In addition, both haemagglutinin and haemolysin are expressed on the membrane of cells infected with measles virus, and nucleocapsid is located intracellularly.¹²⁻¹⁵ Antibodies against haemagglutinin and haemolysin can be identified separately by the functional tests haemagglutination inhibition and haemolysin inhibition. In contrast, the complement fixation test measures the bulk of the antibody to nucleocapsid, apart from a

small proportion of antibody that may be against haemagglutinin.^{12 16 17} Furthermore, we have shown that measles virus haemolysin is sensitive to acetone and that measles virus haemagglutinin is resistant to acetone, and these differences in the sensitivity of the two antigens can be used to measure antibodies to haemolysin and haemagglutinin antigens in the fluorescence antibody test.^{15 18 19} Moreover, in the sera of normal healthy subjects the titres of haemolysin antibodies are always higher than those of haemagglutinin antibodies.^{16 18} Therefore, fluorescence antibody titres obtained by staining the membrane of unfixed cells infected with measles virus represent mostly antibodies to haemolysin and the titres obtained by staining the membrane of acetone fixed cells infected with measles virus represent haemagglutinin antibodies. Similarly, the staining of intracellular inclusions in acetone fixed cells represents antibodies to nucleocapsid.^{15 18 19}

Although both complement fixation and haemagglutination inhibition tests have been used to estimate antibodies specific for measles virus in the sera of patients with chronic active hepatitis, haemolysin inhibition antibodies have not been measured. These antibodies are neutralising¹⁶⁻¹⁸ and are therefore important in protection against measles infection²⁰; they are also found to be in excess of

haemagglutination inhibition antibodies in healthy immune subjects after primary childhood measles.¹⁶⁻¹⁸ In this study we therefore investigated levels of haemolysin and haemagglutinin antibodies specific for measles virus in the sera of a group of patients with chronic active hepatitis using fluorescence antibody and functional tests. We also examined titres of autoantibodies to determine whether there was a correlation between autoantibodies and the viral antibodies.

Material and methods

PATIENTS AND CONTROLS

Serum samples were obtained from 16 patients with chronic active hepatitis negative for HBs Ag. The patients comprised 13 women and three men (mean age 47.7 years; range 27–74 years). The diagnosis was based on the clinical criteria specified by the Fogarty International Centre, 1976,²¹ and on histological criteria described by an international group²² for liver biopsy. At the time of the study seven patients had increased serum aspartate aminotransferase and serum alanine aminotransferase activities (range 60–200 U/l) and nine patients had normal activities (10–45 U/l). All the patients also had increased γ -globulin values. In five cases a second serum sample was obtained after about 11–22 months. Sera were collected over three years and stored at -20°C until tested. All patients were receiving either prednisolone or adrenocorticotrophic hormone, and seven patients were receiving azathioprine in addition.

Control sera were obtained from 16 healthy blood donors (mean age 47.5 years; range 26–74 years). The sera were matched for sex and age within two years.

CELLS AND VIRUS

HEp-2 cell line was obtained from Gibco-Biocult Ltd, and cells were propagated in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and 10% tryptose phosphate broth.

The stock of the Edmonston strain of measles virus adapted to HEp-2 cells was prepared as described previously.²³ Infectivity of the stock pool of virus was measured by plaque assay²³; the titre was 1×10^7 plaque forming units/ml.

HAEMAGGLUTININ AND HAEMOLYSIN ANTIGENS

Extracellular measles virus was used to prepare both haemagglutinin and haemolysin antigens. Monolayers of HEp-2 cells were infected at a multiplicity of infection of 0.01 plaque forming units/cell. Five days after infection, when 90% of the cells showed extensive cytopathic effect, the medium was

removed and clarified by centrifugation at 1700 g for 10 minutes. The supernatant was further concentrated by centrifugation at 73 000 g for two hours at 4°C , and the pellet was resuspended in 0.01M phosphate buffered saline in 1/50 of the original volume.

The haemagglutinin test was carried out in Flow microtitre plates. Serial two fold dilutions (0.025 ml) of the preparation were made in Dulbecco's phosphate buffered saline (pH 7.4), and equal volumes of 1% washed rhesus monkey erythrocytes were added. After incubation at 37°C for one hour the titre of the haemagglutinin was estimated as the reciprocal of the highest dilution that produced complete haemagglutination.

The haemolysin test was carried out in three $\times$ half inch plastic tubes. Serial two fold dilutions (0.25 ml) of the preparation were made in Dulbecco's phosphate buffered saline (pH 7.4), and equal volumes of washed 2% rhesus monkey erythrocytes were added. After incubation at 37°C for 18 hours the samples were centrifuged at 200 g for five minutes and the supernatants examined for haemolysis. The reciprocal of the highest dilution that produced complete lysis was taken as the titre of the haemolysin preparation.

The preparations used in the serological tests had haemagglutinin and haemolysin titres of 1024 and 512, respectively, and single preparations of each antigen were used throughout the study.

SEROLOGICAL METHODS FOR DETECTING ANTIBODIES AND AUTOANTIBODIES SPECIFIC FOR MEASLES VIRUS

To avoid non-specific reactions in the indirect fluorescence antibody method and functional haemagglutination inhibition and haemolysin inhibition tests each serum diluted 1/10 in phosphate buffered saline was first absorbed overnight at 4°C with washed, pelleted HEp-2 cells treated with acetone (30% v/v). After clarifying by centrifugation at 10 000 g for 20 minutes each serum was further absorbed for one hour at 37°C with washed rhesus monkey erythrocytes (obtained from rhesus monkeys kept in the animal house of the Queen's University of Belfast) in a final concentration of 10%. The sera were further clarified by centrifugation at 200 g for 10 minutes before use. The fluorescein isothiocyanate (FITC) conjugated sheep antihuman IgG and FITC conjugated sheep antihuman IgM (Wellcome Diagnostics Ltd, Beckenham, England) used in this study were absorbed with HEp-2 cells treated with acetone as described above. The optimal dilutions of each conjugate were further determined by titration in indirect immunofluorescence tests on cells infected with measles virus, using an

early serum from a patient suffering from the infection. The FITC conjugated sheep antihuman IgG was used at a dilution of 1/60 and the FITC conjugated antihuman IgM at a dilution of 1/30. Only specific staining of cells infected with the virus was observed. The staining of either uninfected or infected cells was not observed with conjugates on their own.

Immunofluorescence Semiconfluent monolayers of HEP-2 cells were grown on 13 mm round coverslips in 5 cm petri dishes (2×10^5 cells per dish) in Eagle's growth medium at 37°C for 18 hours in 5% carbon dioxide. The cells were washed once with Eagle's medium without serum and infected at a multiplicity of 0.01 plaque forming units/cell. After adsorption at 37°C for one hour 4 ml of maintenance medium was added and the dishes were incubated for 48 hours at 37°C to give about 30% infected cells. The coverslips were either used unfixed or were fixed in acetone for eight minutes at room temperature after three washes in phosphate buffered saline and one rinse in fresh acetone. The fixed coverslips were air dried for one hour before use or were stored at -20°C before use. The percentage of infected cells in each preparation was routinely confirmed using FITC conjugated monkey antimeasles serum in the direct immunofluorescence test as described previously.²⁴ The advantage of using this method was that the staining of infected cells could easily be differentiated from that of uninfected cells in the same preparation.

To detect antibodies to measles virus haemolysin serial two fold dilutions of sera in phosphate buffered saline were applied to unfixed coverslip preparations of infected cells that had been rinsed rapidly three times in phosphate buffered saline for 45 minutes at 37°C. After three more quick rinses in phosphate buffered saline FITC conjugated sheep antihuman IgG was applied for 45 minutes at 37°C. After thorough rinsing in phosphate buffered saline the coverslips were mounted in glycerol saline and examined under a fluorescence microscope. The procedure used to detect antibodies to haemagglutinin (including nucleocapsid) was similar to that described for the unfixed preparation, except that instead of quick rinses in phosphate buffered saline the coverslips were washed for 15 minutes in phosphate buffered saline. The fluorescence antibody titre was the highest dilution of the serum that gave specific staining of the membrane of the infected cells for haemolysin antibody and staining of the membrane of the infected acetone fixed cells for haemagglutinin antibody and staining of viral inclusions for nucleocapsid antibody.

Haemagglutination inhibition Serial two fold dilutions of serum were prepared in Dulbecco's phos-

phate buffered saline (pH 7.4) in 0.025 ml volumes in microtitre plates and mixed with a preparation of haemagglutinin containing four haemagglutinating doses. After incubation at 37°C for one hour an equal volume of washed 1% suspension of rhesus monkey erythrocytes was added. The erythrocytes were allowed to settle at 37°C for one hour. The highest dilution of serum giving 50% inhibition of agglutination was taken as the titre of the antibody.

Haemolysin inhibition Serial two fold dilutions of serum were prepared in Dulbecco's phosphate buffered saline (pH 7.4) in 0.25 ml volumes in three × half inch plastic tubes and mixed with a preparation of haemolysin containing eight haemolysing doses. After incubation at 37°C for one hour an equal volume of 2% washed suspension of rhesus monkey erythrocytes was added. After further incubation at 37°C for 18 hours the cells were centrifuged at 200 g for five minutes and the supernatants examined for lysis. The highest dilution of serum giving 50% reduction of haemolysis was taken as the titre of the antibody.

Autoantibodies Unfixed 5 µm cryostat sections of rat liver, kidney, and stomach snap frozen as a composite block with carbon dioxide were used as substrate to detect and estimate smooth muscle antibody, antinuclear antibodies, and antimitochondrial antibodies in IgG and IgM classes. Serial two fold dilutions of sera in phosphate buffered saline were applied to the tissue sections for 30 minutes at room temperature, washed for 45 minutes in phosphate buffered saline and then treated for a further 30 minutes with either FITC conjugated sheep antihuman IgG or FITC conjugated sheep antihuman IgM. After washing for 45 minutes the sections were mounted in glycerol saline and examined under a fluorescence microscope. The titres of autoantibodies were determined according to the criteria described previously.²⁵

ADSORPTION OF SERA WITH SMOOTH MUSCLE TISSUE

A homogenate of smooth muscle tissue was prepared by washing a surgical specimen of human uterus in phosphate buffered saline to remove blood. Four sera from patients with chronic active hepatitis diluted 1/5 were absorbed twice with the homogenate (30% v/v) overnight at 4°C and were clarified by centrifugation at 10 000 g for 15 minutes. The sera were tested by immunofluorescence, in parallel with aliquots of the same specimens unabsorbed for virus specific IgG class of antibody against unfixed antigens, acetone fixed antigens, and autoantibodies in IgG and IgM classes.

Table 1 Geometric mean titres and mean logarithmic titres of antibodies specific for measles virus by immunofluorescence and functional tests

Study group	Titre of virus specific antibodies			
	Immunofluorescence tests (IgG antibody)		Functional tests	
	Unfixed membrane antigens (haemolysin)	Acetone fixed intracellular antigens (haemagglutinin plus nucleocapsid)	Haemolysin inhibition	Haemagglutination inhibition
Chronic active hepatitis (n = 16):				
Geometric mean titre*	1125*	415	1280	247
Mean (SD) logarithmic titre	3.051 (0.514)*	2.618 (0.514)	3.107 (0.440)	2.392 (0.559)
Blood donors (n = 16):				
Geometric mean titre*	190	73	199	44
Mean (SD) logarithmic titre	2.279 (0.356)	1.865 (0.308)	2.298 (0.325)	1.640 (0.379)
Probability†	0.001	0.0007	0.0010	0.0015

*Expressed as reciprocals of serum dilutions.
†Obtained by Wilcoxon matched pairs signed rank test.

STATISTICAL ANALYSIS

The comparison of the virus specific antibody titres in the chronic active hepatitis and control sera were made by the Wilcoxon matched pairs and the signed rank test. The incidence of smooth muscle antibody of IgG class in sera from patients with chronic active hepatitis and controls was evaluated using McNemar's test, and the binomial test was used for comparing the incidence of smooth muscle antibody of IgM class and antinuclear antibodies of IgG and IgM classes in diseased and normal sera. Spearman's rank correlation coefficients were used to test correlations between measles virus specific antibodies to haemolysin (unfixed immunofluorescence) and smooth muscle antibody of IgG class, antinuclear antibodies of IgG class, aspartate aminotransferase,

alanine aminotransferase, and immunoglobulin concentrations in sera of patients with chronic active hepatitis. For comparison of virus specific haemolysin antibody titres (unfixed immunofluorescence) in patients' sera positive for antinuclear antibodies and negative for antinuclear antibodies the Wilcoxon rank sum test was used. The conventional level of significance ($p < 0.05$) was used throughout.

Results

VIRUS SPECIFIC ANTIBODIES

Table 1 shows the geometric mean titres and mean logarithmic titres of measles virus specific antibodies against haemolysin (unfixed) and haemagglutinin

Fig. 1 Reciprocal titres of antibodies specific for measles virus and autoantibodies in 16 patients with chronic active hepatitis (CAH) and 16 controls matched for age and sex.

Table 2 Incidence of IgG and IgM class autoantibodies in patients' and control sera. (Values are numbers (%) of subjects.)

Study group	Immunofluorescence tests					
	IgG class of antibody			IgM class of antibody		
	Smooth muscle antibody	Antinuclear antibodies	Antimitochondrial antibodies	Smooth muscle antibody	Antinuclear antibodies	Antimitochondrial antibodies
Chronic active hepatitis (n = 16)	12 (75)	8 (50)	3 (19)	8 (50)	4 (25)	3 (19)
Blood donors (n = 16)	1 (6)	2 (13)		1 (6)	3 (19)	

(including nucleocapsid) as measured by immunofluorescence and functional tests of inhibition of haemagglutinin and haemolysin. Fig. 1 shows the individual titres of antibodies. The patients with chronic active hepatitis had higher titres of virus specific antibodies as measured by immunofluorescence and functional tests than the normal controls (Table 1). Notably, virus specific IgG antibody against haemolysin by immunofluorescence and haemolysin inhibition tests showed the highest titres. A significant correlation was also found between the virus specific fluorescence antibody test against haemolysin (unfixed) and functional haemolysin inhibition antibody test ($r_s = 0.96$, $p = 0.001$). Similarly, highly significant correlations were found between virus specific IgG antibody against haemolysin (unfixed) by immunofluorescence and haemagglutinin including nucleocapsid (acetone fixed) by immunofluorescence ($r_s = 0.92$, $p = 0.001$).

AUTOANTIBODIES

Table 2 shows the incidence of IgG and IgM classes of smooth muscle antibody, antinuclear antibodies, and antimitochondrial antibodies. Fig. 1 gives the individual titres of these antibodies. A significantly higher incidence of smooth muscle antibody of IgG class ($\chi^2 = 7.78$; $df = 1$; $0.01 > p > 0.001$) and smooth muscle antibody of IgM class ($p = 0.04$) was found in the patients' sera, and the titre of smooth muscle antibody was higher in the IgG class than in

the IgM class. Although the incidence of antinuclear antibodies of IgG class was greater in patients' sera, the difference was not significant ($p = 0.07$). This may have been due to the small number of patients in the study group. No difference was observed in the incidence of antinuclear antibodies of IgM class between the patient and control groups. Antimitochondrial antibodies of IgG and IgM classes were found only in a small number of patients' sera and not in control sera (Table 2).

VIRUS SPECIFIC ANTIBODIES AND AUTOANTIBODIES IN DUPLICATE SERA

Table 3 shows the comparison of the virus specific antibodies and autoantibodies in the duplicate sera obtained from five patients after 11–24 months. The results indicated that both the virus specific antibodies and the autoantibodies fluctuated, but as the number of patients was small statistical analysis was not carried out.

CORRELATION BETWEEN VIRUS SPECIFIC ANTIBODIES AND AUTOANTIBODIES

In patients with chronic active hepatitis a highly significant correlation was observed between virus specific haemolysin antibody (unfixed) and smooth muscle antibody of IgG class ($r_s = 0.78$, $p = 0.001$; Fig. 2), whereas the correlation with smooth muscle antibody of IgM class was less significant ($r_s = 0.54$, $p = 0.03$). A significant correlation was not found between virus specific haemolysin antibody and

Table 3 Comparison of titres* of antibodies specific for measles virus and autoantibodies in duplicate serum samples obtained from five patients

Case No	Interval between samples (months)	Sample	Fluorescence antibody IgG class		Functional antibody		Autoantibodies IgG class	
			Unfixed antigens	Acetone fixed antigens	Haemolysin inhibition	Haemagglutination inhibition	Smooth muscle antibody	Antinuclear antibodies
1	11	First	2560	1280	2560	1280	640	20
		Second	1280	640	1280	320	40	<10
2	11	First	160	40	160	20	<10	<10
		Second	320	80	640	40	<10	<10
3	22	First	1280	320	1280	160	<10	<10
		Second	320	160	320	80	20	<10
4	18	First	160	40	160	20	40	<10
		Second	320	80	640	40	<10	<10
5	11	First	2560	2560	2560	640	<10	<10
		Second	2560	1280	2560	640	80	<10

*Expressed as reciprocals of serum dilutions.

Fig. 2 Correlation between titres (unfixed immunofluorescence) of haemolysin antibody specific for measles virus and smooth muscle antibody titres in 16 patients with chronic active hepatitis ($r_s = 0.78$).

antinuclear antibodies of IgG class ($r_s = 0.19$, $p = 0.48$). This lack of correlation was further confirmed by comparing titres of virus specific haemolysin antibody in sera with and without antinuclear antibodies ($p = > 0.10$). Significant correlations were not found between virus specific haemolysin antibody and serum aspartate aminotransferase and alanine aminotransferase values ($r_s = 0.18$, $p = 0.5$; $r_s = -0.01$, $p = 0.96$) and total immunoglobulin values ($r_s = 0.23$, $p = 0.44$).

SERUM ABSORPTION

Absorption of four sera from patients with chronic active hepatitis with smooth muscle tissue prepared from human myometrium either removed completely or reduced the titres of smooth muscle antibody, antinuclear antibodies, and antimitochondrial antibodies of both IgG and IgM classes, but the titres of the virus specific IgG antibodies against haemolysin (unfixed) and haemagglutinin including nucleocapsid (acetone fixed) were not affected.

Discussion

Increased antibody titres to the envelope antigen haemagglutinin and the internal antigen nucleocapsid of measles virus have been shown previously in the sera of patients with chronic active hepatitis, using haemagglutination inhibition and complement fixation tests. In this study we have shown raised levels of antibodies not only to haemagglutinin but also to the second envelope antigen haemolysin. The antibody titres to haemolysin antigen were found to be higher than antibodies to haemagglutinin by both

fluorescence antibody and functional tests, and the significant correlation between these two methods supports our observations. Similar increased titres to measles haemolysin have been reported in patients with subacute sclerosing panencephalitis, multiple sclerosis, and rheumatoid arthritis.¹⁶⁻¹⁸

Our findings of the incidence of autoantibodies in sera of patients with chronic active hepatitis agree with previous reports.⁸⁻¹⁰ Although the incidence of smooth muscle antibody of IgG class and smooth muscle antibody of IgM class was significantly higher in patients with chronic active hepatitis, by far the highest incidence with high titres was observed in smooth muscle antibody of IgG class, which is accepted as a useful autoantibody marker in patients with chronic active hepatitis.⁸⁻¹⁰ The significant correlation observed between smooth muscle antibody of both IgG and IgM classes with virus specific haemolysin antibody is interesting because in patients with chronic active hepatitis smooth muscle antibody of IgG class shows actin specificity,²⁵ and it has also been shown that actin is incorporated into measles virion.¹³ In contrast, smooth muscle antibody of IgM class reacts with cellular intermediate filaments.²⁵ The absorption of the sera with smooth muscle tissue prepared from human myometrium did not affect the titres of the virus specific haemolysin or haemagglutinin antibodies but it either reduced or completely removed the smooth muscle antibody of IgG class. These results are not surprising because the smooth muscle used for absorption was a crude preparation that contained all the contractile proteins.²⁶ Therefore, if there were restricted but common antigenic determinants shared by measles virus and actin of smooth muscle tissue this absorption would not be effective.

It has been reported recently that monoclonal antibodies against the protein of intermediate filaments of human cells cross react with phosphoprotein of both measles virus and herpes simplex virus. In contrast, monoclonal antibodies against measles virus phosphoprotein did not react with the phosphoprotein of herpes simplex virus and vice versa, indicating recognition by monoclonal antibodies of different antigenic determinants on intermediate filaments.²⁷ Further studies with monoclonal antibodies to different antigenic determinants of actin will help to elucidate the correlation observed between smooth muscle antibody of IgG class and haemolysin antibodies specific for measles virus in patients with chronic active hepatitis.

The incidence of antinuclear antibodies of IgG class was higher in the patients with chronic active hepatitis compared with the control group, but there was no significant correlation between virus specific haemolysin antibody and antinuclear antibodies of

IgG class, contrary to the observations reported by other workers.⁴⁻⁶ This difference may have been due to the fact that in other studies the complement fixation test was used to measure antibodies specific for measles virus, whereas in our investigation we examined the correlation between envelope haemolysin antigen and antinuclear antibodies of IgG class. It is also important to emphasise that the use of a single method—namely, immunofluorescence—for assay of both measles virus specific antibodies and autoantibodies and their subsequent correlation has an added advantage. Anti-mitochondrial antibodies, which are considered to be a valuable autoantibody marker in patients with primary biliary cirrhosis but not in those with chronic active hepatitis,⁸⁻¹⁰ were detected in only a few patients.

The implications of raised levels of antibodies against measles virus envelope antigens and of autoantibodies in patients with chronic active hepatitis are unclear. A role for measles virus in the pathogenesis of chronic active hepatitis has been postulated,³⁻⁷ but so far there is no convincing evidence for the persistence of measles virus antigens or measles virus genome in patients with chronic active hepatitis, nor is there evidence of reinfection in these patients. Similar increased titres of haemolysin inhibition and haemagglutination inhibition antibodies specific for measles virus have also been found in other diseases of unknown aetiology such as multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus^{18-28,29} and probably reflect defects in immunoregulatory mechanisms. For instance, lack of suppressor T lymphocyte function has been reported in all these diseases³⁰⁻³² and also in chronic active hepatitis.³³ In view of the fluctuations in titres of both measles virus antibodies and autoantibodies in duplicate sera, further comparative studies on sequential samples from patients with chronic active hepatitis of virus specific antibodies and the factors that help regulate the immune system would be of value.

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