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Research article

Lack of autoantibody production associated with cytomegalovirus infection

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Abstract

To confirm an association between cytomegalovirus (CMV) infection and the presence of antibodies to Smith (Sm), to ribonucleoprotein (RNP), and to a component of the U1 ribonucleoproteins (U1-70kD), we measured antibodies to these protein antigens using an enzyme immunoassay and an immunoblot. The antibodies were measured in the sera of 80 healthy subjects, one-half of whom were naturally CMV seropositive and one-half were CMV seronegative, and in eight subjects immunized with a live attenuated strain of CMV. None of the vaccinees developed antibodies to Sm, to RNP, or to U1-70kD at either 4 or 12 months after immunization. Additionally, there was no statistically significant association between levels of antibodies to Sm or to RNP and between sera obtained from vaccinees, natural CMV seropositive individuals, and CMV seronegative individuals. One CMV seropositive serum and one CMV seronegative serum tested positive for antibodies to U1-70kD. These data indicate that neither wild-type infection nor the live-attenuated Towne vaccine frequently induce autoantibody production.

Keywords: autoantibodies, cytomegalovirus, RNP antigen, Sm antigen, U1-70kD

Introduction

Antecedent infection with many different microbes is often associated with the development of autoimmune disease in humans, but the pathogenic mechanisms involved, if any, are unknown. Most of the microbes associated with autoimmune disease have been viruses, particularly cytomegalovirus (CMV), Epstein–Barr virus, and varicella–zoster virus. CMV has been associated with the increased production of rheumatoid factor, antiphospholipid antibodies, cold agglutinins, antimyosin antibodies, anti-endothelial cell antibodies, and antiganglioside antibodies. One study found an increased incidence of anti-CMV antibodies among patients with systemic lupus erythematosus [1–11].

Neutralizing antibodies induced by CMV are directed primarily against the major envelope protein of CMV, glycoprotein B (gB). Antibodies to CMV gB share some homology with rheumatoid factor, thus providing a theoretical relationship between CMV infection and autoimmune disease [12]. An adenovirus–CMV gB construct vaccine administered to mice induced a statistically significant increase in the production of antibodies to U1-70kD antibody in both normal and autoimmune-prone mice [13]. Newkirk *et al*. recently reported an increased incidence of antibodies to Sm antigen and antibodies to ribonucleoprotein (RNP) among naturally CMV-infected individuals, as well as an increase in antibodies to U1-70kD [14].

To confirm the findings of Newkirk *et al*. [14], we evaluated sera from individuals either naturally infected with CMV or immunized with the live attenuated Towne strain of CMV for the presence of antibodies to three antigens: Sm, RNP, and U1-70kD. We also assessed the correlation between production of antibodies to gB and antibodies to Sm or RNP.

Methods

Subjects

Anonymously coded serum specimens had been stored at −80°C. These were preimmunization screening sera from 80 normal healthy adult females who volunteered for

CMV = cytomegalovirus; EIA = enzyme immunoassay; gB = glycoprotein B; OD = optical density; RNP = ribonucleoprotein; Sm = ribonucleoproteins recognized by antibodies from a patient named Smith; U1-70kD = component of the U1 ribonucleoproteins.

a Towne vaccine study. Forty naturally seropositive and 40 seronegative sera were used. Subjects were aged between 20 and 53 years (the ages of four individuals were not recorded). Also included were postimmunization serial sera from eight normal healthy women who had received 6000 plaque forming units of the live attenuated Towne vaccine as a single subcutaneous injection. Following immunization, all eight subjects developed antibodies to CMV and to CMV gB. Seventy-five per cent of the CMV seropositive subjects and 85% of the CMV seronegative subjects were Caucasian; the remainder were Afro-American.

Screening for anti-CMV antibodies

Sera were tested for the presence or absence of IgG antibodies to CMV by either latex agglutination (CMVScan; Becton Dickinson, Sparks, MD, USA) or by enzyme immunoassay (EIA) as previously described [15].

Detection of antibodies against Sm and RNP

An indirect, noncompetitive EIA was used for both Sm and RNP antigens to detect IgG antibodies. Microplate wells coated with antigen bound human antibody, which was subsequently bound by an enzyme-labeled conjugate antibody and quantitated colorimetrically (Varelisa; Pharmacia & Upjohn, Freiburg, Germany). Sera were diluted 1:101 for both assays.

The Sm antigen used in this assay was purified from calf thymus. The human recombinant RNP antigens used included the U1-70k, U1A, and U1C antigens. For both Sm and RNP, specific quantitative values for each specimen were obtained by extrapolation of optical densities (OD) from a standard curve derived from six points. For Sm, the negative/positive cutoff value was 10 IU/ml serum or OD = 0.52. For RNP, the negative/positive cutoff value was 5 IU/ml serum or OD = 0.32.

Detection of antibodies to U1-70kD

To detect the presence of IgG antibodies to the U1-70kD ribonuclear protein, both immunoblotting and EIA methods were used as described previously [16–18]. Each sample was tested by immunoblot against Jurkat cell lysates with a 1:100 dilution of sera, and by EIA against a bacterially produced U1-70kD fusion protein that comprised residues 1–205 of u1-70kD. All EIA assays were performed using a serum dilution of 1:1000 and were run taking the average OD of duplicate wells. EIA results were repeated for any samples where the OD of the duplicate wells varied by more than 0.05, and for all samples with positive results by either EIA or immunoblot. In cases where discrepant results were obtained between immunoblot and EIA testing, sera were immunoblotted using a more sensitive technique against both intact and apoptotic Jurkat lysates, as previously described [18,19] using sera diluted 1:5000.

Negative immunoblot and EIA results demonstrated the absence of significant titers of IgG antibodies to U1-70kD. Positive results on immunoblot and EIA or a positive result on one of these two tests and a positive immunoblot for apoptotic U1-70kD demonstrated the presence of antibodies to U1-70kD. A positive immunoblot result that was not confirmed by EIA or follow-up immunoblot would probably reflect recognition of an antigen other than U1-70kD with similar electrophoretic motility (i.e. a negative result). An isolated positive EIA was an indeterminate finding; the weaker the recognition, the less likely it was to be valid. A positive EIA result was an OD value above 0.100. If either the EIA or the immunoblot produced positive results, the more sensitive apoptotic assay was used to verify the presence of antibodies to U1-70kD. The sensitivity of these assays has been previously established [16–19].

Detection of antibodies to gB

Quantitative levels of antibodies against CMV gB were measured by EIA in all seropositive sera as previously described [20]. The OD value obtained for the 1:1600 dilution for each serum was used for statistical calculations. The gB antigen used in this assay was a recombinant derivative of human CMV strain Towne gB produced as a secreted protein in Chinese hamster ovary cells [21]. The recombinant gB includes amino acids 1–676 of the extracellular domain. The proteolytic cleavage site at amino acid 437 was blocked by the site-specific mutation of amino acid residues 433, 434, and 436 [22].

Statistical calculations

Comparisons were carried out using Student's *t* test or chi-square analysis. Regression analysis was performed using Sigma Plot (version 1.02; Jandel Corporation, San Rafael, CA, USA).

Results

Antibodies against Sm and RNP

Using the manufacturer's sera to establish a negative/positive cutoff value, none of the sera tested contained detectable levels of antibodies to either Sm or RNP (Table 1). For Sm, using the mean OD plus two standard deviations (Table 1) of the 40 CMV seronegative sera to establish a negative/positive cutoff value, none of the 40 CMV seropositive sera were positive, one of the CMV seronegative sera was positive (OD = 0.422), and none of the sera from the vaccine recipients were positive. For RNP, using the mean OD plus two standard deviations (Table 1) of the 40 CMV seronegative sera to establish a negative/positive cutoff value, two of the 40 CMV seronegative sera were positive (OD = 0.22 and 0.30), three of the CMV seropositive sera were positive (OD = 0.25, 0.26 and 0.25), and none of the sera from the vaccine recipients were positive.

Table 1

Association between cytomegalovirus (CMV) infection and antibodies to Smith (Sm) and to ribonucleoprotein (RNP)

Subjects	Sm antigen	RNP antigen
CMV negative	0/40(0.067 ± 0.143)	0/40 (0.116 ± 0.091)
CMV positive	0/40 (0.067 ± 0.095)	0/40 (0.128 ± 0.097)
CMV vaccinees		
Preimmunization	0/8 (0.055 ± 0.051)	0/8 (0.114 ± 0.036)
4 months postimmunization	0/8 (0.056 ± 0.032)	0/8 (0.117 ± 0.041)
12 months postimmunization	0/8 (0.050 ± 0.028)	0/8 (0.111 ± 0.048)

Data presented as number positive/total* (mean optical density ± two standard deviations). * The negative/positive cutoff value used was established by standard sera provided by the manufacturer (see text).

Table 2

Association between antibody levels to cytomegalovirus (CMV) glycoprotein B (gB) and antibody levels to Smith (Sm) and to ribonucleoprotein (RNP) in seropositive sera

		Statistical correlation with optical density to:					
		Sm			RNP		
Subjects	EIA optical density to gB (mean ± SD)	*R*	*t*	*P*	*R*	*t*	*P*
CMV seropositive ($n = 40$)	1.152 ± 1.01	0.118	0.735	0.234	0.214	1.351	0.092
Vaccinees ($n = 8$)							
4 months postimmunization	1.479 ± 2.46	0.147	0.332	0.376	0.147	0.365	0.364
12 months postimmunization	0.690 ± 1.18	0.571	1.702	0.070	0.062	0.153	0.442

EIA, enzyme immunoassay; SD, standard deviation.

To determine whether there was a statistically significant association between levels of antibodies to CMV gB and the levels of antibodies to Sm antigen or RNP antigen, a simple linear regression analysis of gB OD values versus Sm and RNP OD values for sera from CMV seropositive subjects and for sera from vaccines at 4 and 12 months after immunization was performed. No significant correlations were found (Table 2).

Antibodies against U1-70kD

Using the EIA with U1-70kD as the antigen, only one of 104 sera tested was positive (OD = 0.121). That one serum was negative using an immunoblot with apoptotic Jurkat cells. Using an immunoblot, three of 104 sera were positive and three sera were weakly positive. None of the three weakly positive sera were positive using an immunoblot with apoptotic Jurkat cells, but two of the three sera positive by immunoblot were also positive using an immunoblot with apoptotic Jurkat cells. No sera was positive both by immunoblot and by EIA. There was no significant difference for the rate of positivity between sera obtained for CMV seropositive subjects and CMV seronegative subjects (Table 3). None of the recipients of CMV vaccine developed antibodies to U1-70kD (Table 3).

Discussion

The present study was designed to confirm the report of Newkirk *et al*. They reported that, among the sera of 100 normal healthy adults (50 CMV seropositive and 50 CMV seronegative), 54% contained antibodies to RNP, 50% contained antibodies to Sm, and 33% contained antibodies to U1-70kD [14].

Table 3

Association between cytomegalovirus (CMV) infection and autoantibodies to a component of the U1 ribonucleoproteins (U1-70kD)

Subjects	Number positive*/total
CMV seronegative	1/40
CMV seropositive	1/40
Vaccinees	
Preimmunization	0/8
4 months postimmunization	0/8
12 months postimmunization	0/8

* All sera positive were positive by immunoblot (see text).

Newkirk *et al*. also observed that the frequency of autoantibodies to each of the antigens occurred more frequently among CMV seropositive subjects than among CMV seronegative subjects[14]. For CMV seropositive subjects, they observed that 42 (84%) subjects had antibodies to RNP, 32 (64%) had antibodies to Sm, and 23 (46%) had antibodies to U1-70kD [14]. If Newkirk *et al*. used a negative/positive cutoff value of the mean plus three standard deviations then, overall, less than 10% of their sera contained autoantibodies.

We could not reproduce the data of Newkirk *et al*. The subjects in the study of Newkirk *et al*. were similar to our subjects; 80% female and 98% Caucasian. Although there are only a few published reports on the frequency of these antibodies in normal populations, those published reports all find a frequency of between 0 and 3%, similar to those reported in the present study [23–27]. One study of over 1000 healthy pregnant and nonpregnant Israeli women found that none had IgG antibodies to either Sm or RNP. IgM antibodies, however, were detected in 4% or less of subjects. Patients with autoimmune disease have predominantly IgG antibodies to Sm and to RNP, and to a lesser extent IgM antibodies, whereas patients with inactive autoimmune disease are most likely to have IgM antibodies to these antigens [28,29]. Both the present study and that of Newkirk *et al*. measured IgG antibodies to these nuclear antigens.

Several factors may account for the difference between our results and those of Newkirk *et al*. Differences in assay methods or antigens could be important. This is suggested by the fact that the mean OD (>0.5) observed by Newkirk *et al*. in their Sm and RNP EIA assays was significantly higher than the mean OD (<0.15) observed in the present study. Another possibility relates to the negative/positive cutoff value used. For all three antigens, Newkirk *et al*. used EIA assays and established their negative/positive cutoff value using the mean plus two standard deviations of 15 CMV seronegative sera [14]. This appears to have resulted in a negative/positive cutoff value significantly lower than that observed in the present study using either the manufacturer's recommended cutoff value or our own cutoff value established with the 40 seronegative sera. To detect antibodies to U1-70kD, Newkirk *et al*. used only an EIA assay. Using the EIA assay, we found only one of 104 sera contained antibodies to this protein.

Another factor that may account for the difference between our results and those of Newkirk *et al*. is the prevalence of the HLA antigen DR4. This HLA type occurs among 60% of patients with autoimmune disease and antibodies to U1-70kD, but its prevalence in the normal healthy individuals is only about 25% [16,30]. Hence, if the association between HLA DR4 and the presence of antibodies to U1-70kD exists for healthy individuals and if, due to selection bias, our population contained very few ($<4\%$) DR4-positive individuals and the population of Newkirk *et al*. contained a very high ($\geq 50\%$) prevalence of DR4-positive subjects, this could account for the observed differences. This possibility, however, seems very improbable.

In another study, Newkirk and coworkers also observed that a recombinant gB vaccine, which expressed the gB protein of the Towne vaccine, induced antibodies to CMV gB when administered to mice, suggesting that CMV gB induces antibodies crossreactive to U1-70kD [13]. If this is the case, it predicts a correlation between levels of antibodies to gB and U1-70kD in sera. In humans, neither the present study or that of Newkirk and colleagues [13] found such a correlation. This indicates that either there is no such crossreactivity or that, if it exists, it occurs very infrequently or only to a few epitopes. It is also possible that the mice Newkirk and coworkers used were genetically primed to produce autoantibodies in response to this antigen.

Whether viruses cause autoimmune disease is controversial. If they do cause disease, several mechanisms may explain the association between viruses and autoimmune disease. To stimulate a complete autoimmune response, two signals (one antigen specific and one not antigen specific), are necessary [31]. The best described antigen-specific mechanism is molecular mimicry, whereby some component of the offending virus resembles the host structure on a molecular level, thus providing the template for antibody formation that may crossreact with self-antigen. Several of the nonantigen-specific signals include costimulatory cell surface markers as well as the generation of a multitude of cytokines. Theoretically, viruses may play a role in eliciting either or both of these signals.

Infection with CMV is ubiquitous within the human population, and nearly 100% of humans eventually acquire a CMV infection. On the contrary, autoimmune disease is relatively rare, occurring in less than 5% of the population. If CMV was a frequent inducer of autoantibodies, and by implication an autoimmune disease, both the frequency of autoantibodies in disease-free individuals and the incidence of autoimmune disease in the general population would be much higher than observed by other workers and ourselves. It is not excluded, however, that a low frequency of these three autoantibodies may be infrequently but significantly associated with CMV infection. To establish this will require testing of a large number of sera. For example, testing of nearly 700 sera will be required to determine whether an autoantibody frequency of 5% among CMV seropositive individuals and of 1% among CMV seronegative individuals is a significant difference.

Conclusion

We failed to detect antibodies to either Sm or RNP in individuals infected with wild-type CMV or in eight individuals

vaccinated with the Towne strain of CMV. Likewise, regression analysis of levels of antibodies to CMV gB, the major antibody formed after natural infection or active immunization, failed to demonstrate a correlation with the levels of antibodies to Sm and to RNP. With regards to antibody to U1-70kD, which may be a more sensitive indicator of autoimmune disease, the sera from only one CMV seropositive subject contained these antibodies and none of the sera of the vaccinees contained these antibodies. These results indicate that CMV infection induces these autoantibodies infrequently and that autoimmune disease associated with CMV infection is probably rare.

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