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Relationship between cytomegalovirus and colonization of the oropharynx by Gram-negative bacilli following renal transplantation

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SUMMARY

Numerous investigators have reported an increased incidence of pneumonia caused by Gram-negative bacilli and other secondary pathogens in transplant recipients infected by cytomegalovirus (CMV). To determine if CMV infections are related to colonization of the upper respiratory tract by Gram-negative bacilli, we examined prospectively 22 renal transplant recipients with sequential bacteriological, virological and biochemical examinations performed just prior to and at various times after transplantation. Only 11% of subjects had Gram-negative bacilli isolated from gargle specimens prior to transplantation, as compared to 54% after transplantation. More importantly, after transplantation, subjects with active CMV infections were more likely to have prolonged oropharyngeal carriage of Gram-negative bacilli than subjects without CMV infections (36% *v.* 25%). During active CMV infections, the rate at which Gram-negative bacilli were isolated from gargle specimens rose from 28 to 47%. During culture-positive CMV infections, the isolation rate reached 57% and was significantly different from that of CMV-negative samples ($P < 0{\cdot}01$). The increased rate of Gram-negative bacillary isolation from gargle specimens during CMV infections was not a function of type of immunosuppresive agents used, rejection episodes, antibiotic administration, concomitant hepatitis B, Epstein–Barr (EBV) virus, or herpes simplex virus infections, or alterations in salivary fibronectin concentrations.

INTRODUCTION

Transplant recipients, AIDS patients, and patients with a variety of other immune deficiency disorders have a predilection for cytomegalovirus (CMV) infections [1, 2]. In addition to the morbidity and mortality which results directly from these infections, CMV predisposes to secondary infections [3–5]. These superinfections are caused by various microorganisms, including Gram-negative

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bacilli, fungi and protozoa, whose only common characteristic, other than their association with CMV, is a propensity for localization within the respiratory system.

Extensive research has been devoted to unravelling the mechanisms involved in such superinfections. These efforts have focused primarily on the effects of CMV infection on the immune response and, in fact, have established that CMV infection subverts this response in various ways that could limit the host's capacity to combat secondary pathogens [6, 9]. Little, however, has been learned about the initial phase of such secondary infections, the phase during which potential pathogens first establish a presence in the host. It is not known if CMV infection promotes colonization by such pathogens, and in so doing, helps consummate the first phase of invasion by these pathogens; nor is it known why secondary infections in CMV-infected patients primarily affect the respiratory system.

Because a critical initial step in the pathogenesis of acute bacterial infections of the respiratory tract is colonization of the upper airways by potential pathogens [10, 11], we have examined the relationship between CMV infection and colonization of the oropharynx by Gram-negative bacilli in a prospective, longitudinal survey of renal transplant recipients. In initiating this investigation, we reasoned that if CMV infection predisposes transplant recipients to Gram-negative bacillary pneumonia, as opposed simply to increasing the severity of such infections, then CMV infection must promote colonization of the oropharynx by Gram-negative bacilli.

PATIENTS AND METHODS

Subjects

Twenty-two patients were recruited from the Renal Transplant Clinic at the Parkland Memorial Hospital between July 1985 and January 1987. Patients were examined using assays described below prior to receiving their transplant and then at the first, second, third, sixth, and ninth month after transplantation. In addition to the assays described below, routine blood chemistry determinations were performed at the time of each study visit. Clinical characteristics of the subjects are summarized in Table 1.

Virological techniques

Surveillance viral cultures of urine, throat washings, and buffy coat were performed at each sampling time. This was accomplished by inoculation of tubes of human embryonic lung cells (HEL cells, Abbott Laboratories, Abbott Park, Illinois) with both filtered and unfiltered material and observing monolayers for 6 weeks for characteristic cytopathic effect (CPE).

In addition, antibody to CMV and Epstein–Barr virus (EBV) was measured in serum specimens obtained at the time of each study visit. Antibody to CMV was measured in a microtitre complement fixation (CF) assay using commercially prepared CMV-CF antigen (Whittaker-Bioproducts, Inc., Walkersville, MD). Antibody to EBV viral capsid antigen was quantified using an indirect fluorescent antibody assay (Electro-Nucleonics, Inc., Columbia, MD). Hepatitis B surface

Table 1. *Clinical characteristics of the 22 study subjects*

Age (±S.D.)	37·6±10·4 years
Sex (M:F)	16:6
Primary renal disorder [No. (%)]	
Hypertension	8 (38)
Diabetes	5 (24)
Intravenous drug abuse	3 (14)
Chronic glomerulonephritis	2 (10)
Structural defect	4 (20)
Immunosuppressive therapy	
Cyclosporin	17 (81)
Prednisolone	21 (85)
Azathioprim	10 (48)
ALG	7 (37)
Deaths	1 (5)
Rejection episodes	6 (28)

antigen assays were also performed using the Auszyme Monoclonal Diagnostic kit (Abbott Laboratories, Chicago, Illinois).

Bacteriological techniques

To characterize the oropharyngeal bacterial flora, each subject gargled with 10 ml of sterile saline for approximately 10 seconds and returned the gargle into a sterile container. The gargle specimen was mixed vigorously and then diluted 10-fold serially using saline. One-tenth millilitre samples of the dilutions were spread on blood agar and MacConkey's agar. The plates were incubated at 37 °C for 24 h. By examining plates for characteristic colonies and multiplying the number of colonies seen by the appropriate dilution factor, total aerobic bacteria, α-haemolytic streptococci and Gram-negative bacilli were quantified in each gargle specimen. Bacterial isolates were identified using the TouchScan SR system (American MicroScan, Mahwah, NY).

Fibronectin assay

Fibronectin digestive activity was measured using methods previously described by Dal Nogare and colleagues [12]. Briefly, human fibronectin purified on a gelatin Sepharose 4-B column and labelled with tritiated formaldehyde (New England Nuclear), was placed in 6-mm microtitre wells (Nunc, Kamstrup, Denmark) and dried to a film (at 37 °C for 24 h). For the assay of salivary fibronectin digestive activity, saliva samples were placed in fibronectin-coated wells and incubated with TRIS buffer for 3 h at 37 °C. Supernatants of centrifuged samples from each well were then counted on a β scintillation counter. All samples were tested in duplicate. The c.p.m. reading from the β counter, which represented the release of digested fibronectin peptides, was converted to micrograms of fibronectin digested by the formula: Sample c.p.m. − background c.p.m./specific activity of fibronectin (c.p.m./μg) = μg tritiated fibronectin digested.

Statistical methods

The data were analysed using several methods. To reflect the multivariate nature of the data, a categorical linear model analysis was performed. The CATMOD procedure in the SAS program package (SAS User's Guide: Statistics,

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Version 5 Edition, Cary, NC: SAS Institute Inc., 1985) was used to look for interdependencies. To look for overall associations between events (i.e. the traditional RxC contingency table analysis) the FREQ procedure of SAS was used. Both yielded chi-square tests for significance.

RESULTS

Virological studies

Fourteen subjects (64%) exhibited evidence of active CMV infections 1–9 months (median = 2 months) after transplantation. Five (23%) were primary CMV infections (i.e. infections in initially seronegative subjects), and nine (41%) were reactivation of CMV infections (i.e. infections in patients seropositive for CMV prior to transplantation). Eight (36%) patients had no evidence of active CMV infections following transplantation. Three (14%) were seronegative for CMV. CMV infections were most often asymptomatic. However, four CMV infections were associated with fever and/or other symptoms, and two infections coincided with rejection episodes. Five other rejection episodes were not temporally related to CMV infections.

Other viral infections were less common. Nine (41%) subjects had one or more cultures positive for herpes simplex virus (HSV). Three patients exhibited a $\geqslant$ fourfold rise in antibody titre to EBV during the post-transplant period of observation. No patient exhibited clinical evidence of hepatitis, nor did any of the 18 patients on whom one or more serum hepatitis B surface antigen determinations were performed demonstrate a positive result.

Bacteriological studies

Gram-negative bacilli were isolated from only one of nine (11%) of the subjects examined prior to transplantation (Fig 1). Following transplantation, 12 of 22 (54%) subjects examined had Gram-negative bacilli isolated from the oropharynx on at least one occasion. Six subjects had identical Gram-negative bacilli isolated from two or more consecutive gargle samples over periods of at least 1 month and thus met the definition of Spijkevret and colleagues [13] for oropharyngeal carriers. *Klebsiella pneumoniae* was the most commonly isolated Gram-negative bacillus in these carriers. Subjects with CMV infections following transplantation were more likely to exhibit sustained oropharyngeal carriage of Gram-negative bacilli than subjects without CMV infections (36 *v.* 25%). Three subjects (21%) in the former category, as compared to none (0%) in the latter category, were long-term oropharyngeal carriers of two or more Gram-negative bacilli.

Factors affecting bacterial colonization

Analysis of specimens according to CMV activity revealed Gram-negative bacilli in 47% of oropharyngeal cultures obtained during active CMV infections (i.e. at times of positive CMV cultures or CMV seroconversions) as compared to only 28% of specimens obtained when no evidence of active CMV infection existed (i.e. at times of negative CMV cultures and stable CMV titres) ($P = 0{\cdot}064$). When oropharyngeal cultures obtained during culture-positive CMV infections were

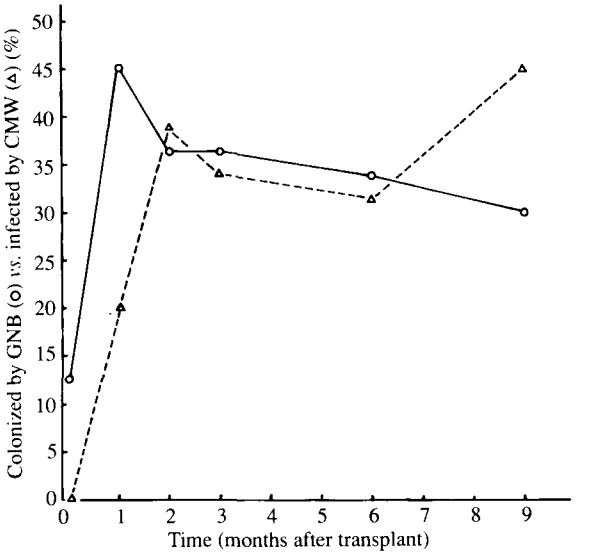

Fig 1. relationship between sampling period and detection of CMV infection (△) and isolation of oropharyngeal Gram-negative bacilli (○).

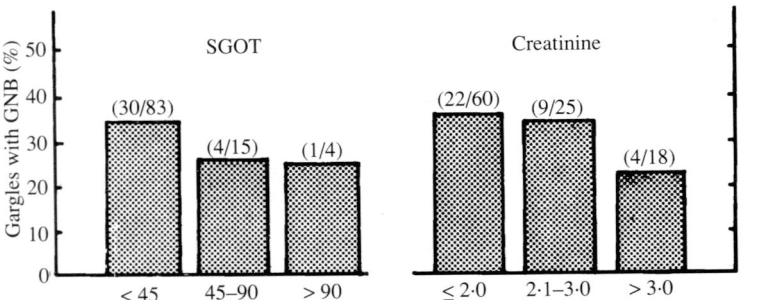

Fig 2. Relationship between the serum creatinine and SGOT and oropharyngeal Gram-negative bacilli (GNB).

compared to those obtained during CMV culture-negative periods, the difference in rates of isolation of Gram-negative bacilli reached statistical significance (57 *v.* 26%; $P < 0{\cdot}01$). In contrast, HSV infections (33% isolation rate) were not apparently associated with oropharyngeal colonization by Gram-negative bacilli.

Colonization of the oropharynx by Gram-negative bacilli was not a sequela of recent antibiotic administration. Twenty-one of the 105 oropharyngeal cultures analysed were obtained during, or within 1 week of, antibiotic therapy. Only six (29%) contained Gram-negative bacilli. The high rate of colonization during the post-transplantation period could not be attributed to rejection episodes. Of the seven specimens obtained during such episodes, only two (28%) contained Gram-negative bacilli. Finally, neither the serum creatinine nor the SGOT correlated directly with Gram-negative bacillary colonization of the oropharynx (Fig. 2).

Oropharyngeal cultures obtained during active CMV infections contained total concentrations of aerobic bacteria (data not shown) and α-haemolytic streptococci (Fig. 3) that differed little if any from those obtained when patients were free from

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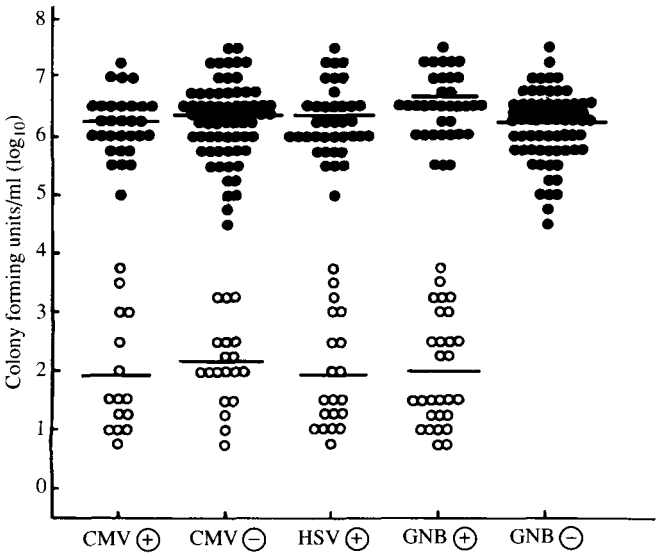

Fig 3. Comparison of the results of quantitative bacterial cultures of saline gargles obtained during active CMV (CMV+) and HSV (HSV+) infections and during CMV-negative periods (CMV−). Concentrations of α-haemolytic streptococci (●); Gram-negative bacilli (○). Results for specimens containing Gram-negative bacilli (GNB+) are also compared with those lacking these bacilli (GNB−). Bars indicate mean values for each data set.

Table 2. *Identity of Gram-negative bacilli isolated from transplant gargle specimens*

Gram-negative bacillus	CMV-positive (31)*	CMV-negative (65)*	HSV-positive (11)*
Klebsiella	9 (29)†	9 (14)†	3 (27)†
Proteus	2 (6)	3 (5)	2 (18)
Escherichia	1 (3)	2 (3)	0 (0)
Enterobacter	3 (9)	5 (8)	0 (0)
Serratia	1 (3)	2 (3)	0 (0)
Pseudomonas	0 (0)	4 (6)	0 (0)
Misc.	4 (13)	2 (3)	1 (9)
⩾ 2 Gram-negative bacilli	5 (16)	8 (12)	2 (18)

* No. specimens examined.
† No. positive specimens (% positive).

active CMV infections or when patients were infected by other viruses. Furthermore, specimens containing Gram-negative bacilli did not differ from those lacking Gram-negative bacilli with respect to concentrations of either total bacteria or α-haemolytic streptococci. Concentrations of Gram-negative bacilli (Fig. 3) and species of Gram-negative bacilli (Table 2) observed during CMV infections did not differ from those observed in colonized subjects at other times. Fibronectin assays showed similar levels of fibronectin digestive activity in saliva obtained during active CMV infections and in saliva obtained at other times (Fig. 4).

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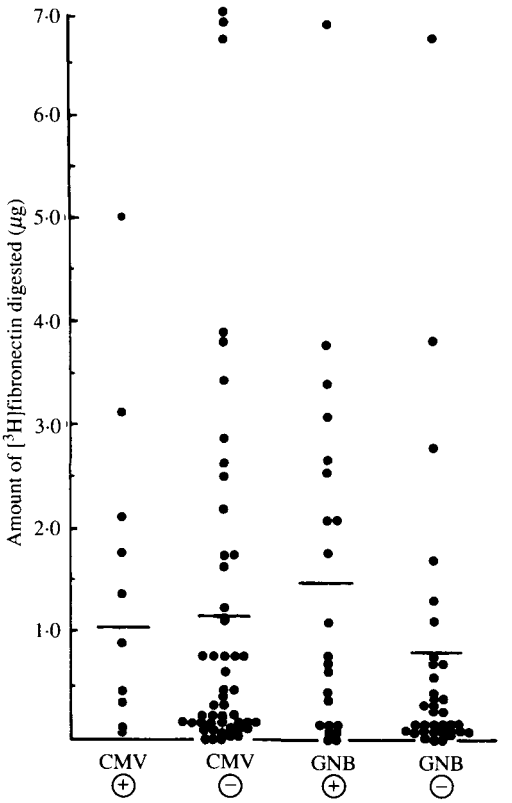

Fig 4. Salivary fibronectin digestive activity in samples obtained during active CMV infections (CMV+) and during CMV-negative periods (CMV−). Results of samples obtained during Gram-negative bacillary colonization (GNB+) are also compared with those obtained when no such colonization existed (GNB−). Bars indicate mean values for each data set.

DISCUSSION

In the present investigation, as in several previous investigations [14–17], CMV infection was the most common viral infection identified in renal transplant recipients following transplantation. Sixty-four percent of our subjects developed CMV infection after receiving renal allografts – 41% had reactivation infections and 23% primary CMV infections. Infections caused by HSV and EBV were also identified, but were less common. Hepatitis B virus infection was not diagnosed in our study population.

Whereas before transplantation, the rate at which Gram-negative bacilli were isolated from gargle specimens (11%) was less than the rate (18%) observed in healthy control subjects in an earlier study using identical sampling techniques [18], after transplantation the isolation rate increased markedly (36%). During the post-operative period, such colonization occurred most often in association with CMV infections. Thirty-six percent of subjects developing CMV infections exhibited prolonged oropharyngeal carriage of Gram-negative bacilli, as opposed to 25% of those not actively infected by CMV after transplantation. This

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difference was even more striking when one considered subjects colonized by two or more species of Gram-negative bacilli. Twenty-one percent of those infected with CMV exhibited prolonged carriage of two or more species of Gram-negative bacilli, as compared to none of the subjects not actively infected with CMV. Forty-seven percent of the oropharyngeal cultures obtained from subjects during CMV infections (as indicated by either positive CMV culture or CMV seroconversion at the time of the oropharyngeal culture) contained Gram-negative bacilli. This figure rose to 57% when only culture-positive CMV infections were considered. Several other viral infections were identified in the study population. However, none exhibited any apparent association with oropharyngeal Gram-negative bacilli.

These observations establish an association between CMV and oropharyngeal Gram-negative bacilli in renal transplant recipients. The current investigation was not designed to test causality. However, if the association between CMV and oropharyngeal Gram-negative bacilli is causal, we believe it more likely that CMV infections potentiate oropharyngeal colonization by Gram-negative bacilli than *vice versa*.

CMV might promote such colonization of the oropharynx by Gram-negative bacilli in several ways. In healthy subjects, non-groupable α-haemolytic (viridans) streptococci contained within the normal oral flora play a pivotal role in excluding Gram-negative bacilli from the oropharynx by means of competitive inhibition involving the elaboration of bacteriocins [19–21]. When inhibitory streptococci are suppressed during antibiotic therapy, oropharyngeal overgrowth by Gram-negative bacilli quickly ensues [19]. In our survey, oropharyngeal bacillary colonization during the post-transplant period was not the consequence of either antibiotic-induced or CMV-induced suppression of inhibitory streptococci. In fact, in our study, Gram-negative bacillary colonization of the oropharynx did not correlate in any way with concentrations of α-haemolytic streptococci in the oropharynx.

Previous investigations have shown that fibronectin, a high molecular weight glycoprotein, covers oral epithelial cell surface receptors for Gram-negative bacilli, thereby preventing adherence of these bacteria to such cells [22, 23]. In severely ill patients, excess proteolytic enzyme activity in oral secretions cleaves fibronectin from cell surfaces [24], thus potentiating adherence of Gram-negative bacteria to buccal epithelial cells and in this way promoting colonization of the oropharynx [25]. In the present investigation, salivary fibronectin digestive activity exhibited no apparent relationship with either CMV infections or oropharyngeal colonization by Gram-negative bacilli.

Cytomegalovirus and other viruses within the herpesvirus group induce Fc receptors in infected fibroblast monolayers [26–28]. Cytomegalovirus-induced Fc receptors have previously been shown to facilitate adherence of antibody-coated microorganisms to CMV-infected cells *in vitro* [29] and might perform a similar function during clinical infections *in vivo*. In the present investigation, we conducted *in vitro* assays for such receptors on buccal epithelial cells obtained from subjects during CMV infections, but were unsuccessful in demonstrating virus-induced Fc receptors in any specimen examined (unpublished observations).

In summary, CMV infections appear to be associated with colonization of the

oropharynx by Gram-negative bacilli following renal transplantation. Whether this association is causal, and if so, what mechanisms mediate the association remain to be determined.

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