Clin. exp. Immunol. (1976) 26, 469–477.

Sequential studies of lymphocyte responsiveness and antibody formation in acute bacterial meningitis

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(*Received* 8 *July* 1976)

SUMMARY

Lymphocyte transformation responses *in vitro* were studied in eight patients with acute bacterial meningitis (in five due to *Neisseria meningitidis*). Sequential studies were done from 24–48 hr after the first symptoms of infection to complete recovery. In all cases lymphocyte transformation was depressed during the acute phases of illness. The responses to microbial antigens were more affected than the responses to mitogens. The course of the lymphocyte responses to the causative micro-organism showed no difference from the responses to other microbial species.

A moderate shift towards increased sensitivity of the lymphocytes to lower doses of the causative micro-organism was observed during the course of illness in three cases.

In *N. meningitidis* infection, a rapid rise was seen in the serum titres of complement-fixing antibodies and in the number of precipitating antibodies, whereas the rise in immunoglobulin concentrations was more prolonged. Characteristic patterns of elevation and return towards normal were found in the serum concentrations of the acute-phase reactants α_1-antitrypsin, haptoglobin, and orosomucoid.

It is concluded that the lymphocyte transformation responses *in vitro* during severe bacterial infection are largely governed by non-specific factors, and that studies of lymphocyte responses to micro-organisms should always include other microbial species as controls.

INTRODUCTION

During acute bacterial infections, cellular immune functions are depressed (Kantor, 1975). Sequential studies in patients, comparing the lymphocyte response to the causative micro-organism with the responses to other microbial antigens, are needed to define the extent and mechanisms of this depression. In the present report, acute bacterial meningitis was chosen for study, because of the rapid and well-defined onset of symptoms and the prompt response to therapy in uncomplicated cases. This made it possible to follow selected aspects of immune function at close intervals, from shortly after the first manifestations of infection to complete recovery.

MATERIALS AND METHODS

Patients. Eight patients with acute bacterial meningitis were studied; detailed clinical information, in particular concerning the treatment given, is presented in the companion article (Hansen *et al.*, 1976). Time zero is defined as the onset of the first symptoms. The first blood sample was drawn at 24–48 hr. Further sampling was done initially three times weekly, during recovery at greater intervals until discharge from hospital, giving a total of six to eleven samples per patient. For quantitation of serum proteins, samples were taken once a week. Five patients were re-studied approximately 1 year after discharge, when they were in good health.

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Microbial preparations. Culture of the cerebrospinal fluid revealed the pathogen in all cases: *N. meningitidis* (five patients), *Streptococcus pneumoniae* (two patients), and *E. coli* (one patient). Four out of five *N. meningitidis* strains belonged to the serological group A. It was not possible to employ the patient's own infecting micro-organism in the lymphocyte transformation studies, since the first lymphocyte cultures were set up immediately after the patient's arrival to the hospital. In these studies were employed a randomly selected group A meningococcal strain, a pneumococcal strain of capsular type 19 F, and an *E. coli* strain O 21:H 27.

The bacteria were grown on blood agar plates. They were scraped off the plates, washed three times and resuspended in saline, at a density of 5×10^8 bacteria/ml. They were killed by heating to 70°C for 30 min.; this was necessary to ensure sterility. These heat-killed whole bacteria were added directly to lymphocyte cultures; it has been shown for *Pseudomonas aeruginosa* (Høiby, Andersen & Bendixen, 1975) and for *Bordetella pertussis* (Andersen *et al.*, 1976) that whole bacteria are more efficient stimulators of lymphocyte transformation than are soluble extracts prepared by sonication.

Other microbial preparations employed in the lymphocyte transformation test were an extract of *Candida albicans* (kindly donated by Dr Nils Axelsen) and PPD (Statens Seruminstitut).

Lymphocyte culture. Blood mononuclear cells were separated on Ficoll–Isopaque, washed twice, and resuspended in RPMI 1640 containing 20% pooled AB-serum from healthy, young, non-transfused male donors. 10^5 cells were cultured per vial of 500 μl, in 5% CO_2. All cultures were set up in triplicate. 50 μl bacterial suspension were added per culture. A dose-response relationship was established at each sampling, by adding four-fold dilutions of bacteria. Possible toxicity for lymphocytes was studied in replicate cultures by the addition of phytohaemagglutinin (PHA) after 24 hr culture.

As mitogens were employed PHA, concanavalin A (Con A), and pokeweed mitogen (PWM). Details of the culture technique, and the mitogens and antigens employed have been described (Andersen, Thomsen & Cohn, 1975). Cultures stimulated by mitogen were harvested at 72 hr, antigen-stimulated cultures after 120 hr. 0·05 μCi [^{14}C]thymidine was added 24 hr before termination of culture. Thymidine incorporation was quantitated by liquid scintillation counting. The results are given as ct/min.

Quantitation of anti-bacterial antibodies in serum. Sera were stored at −20°C and studied simultaneously at the end of the sampling period.

The preparation of antigen and the complement fixation test employed to measure anti-meningococcal antibodies was analogous to that described for the gonococcal complement fixation test (Reyn, 1965). The series of samples from each patient was tested both with antigen prepared from the causative strain and with a standard preparation made up from a pool of ten strains. The results are given as $\log_{10}$ to the reciprocal value of the lowest serum concentration giving 60% haemolysis.

The technique for determination of precipitating antibodies by crossed immunoelectrophoresis has been described (Høiby & Wiik, 1975; Høiby, 1975); as antigen preparation, the supernatant from bacteria disintegrated by ultrasonication was employed.

Quantitation of serum proteins and blood leucocytes. Serum concentrations of IgG, IgA, IgM, of the acute-phase reactants orosomucoid, α_1-antitrypsin, haptoglobin, and of albumin were quantified by rocket immunoelectrophoresis (Weeke, 1973). The determinations were made in duplicate (coefficient of variation < 5%), and the results were expressed in g/l by comparison with a standard serum from Behringwerke, Lahn, West Germany.

Blood concentrations of lymphocytes and monocytes were established by leucocyte counting, employing standard laboratory methods, and a differential count of 300 leucocytes in May–Grünwald–Giemsa-stained smears.

RESULTS

Lymphocyte stimulation in vitro

The results of lymphocyte transformation studies in a patient with rapid and uncomplicated recovery from *N. meningitidis* infection are shown in Fig. 1. Initially the lymphocyte responses are low. Among the mitogens, the Con A response is more depressed than the responses to PHA and PWM; the responses to microbial antigens are almost extinct at first sampling, and they remain low for a longer period than the mitogen responses. During recovery, the lymphocyte responses improve to values within the range observed in healthy adults.

Fig. 2 depicts the results in a patient whose clinical course was complicated by arthritis. The fluctuations in the PHA response might be considered fortuitous; however, clear changes are seen in the PWM and Con A responses and are even more obvious in the responses to the microbial antigens. The periods of depressed responses were those with recurrence of fever.

The results obtained in all patients studied are quantified in Table 1. It appears from these results that the response to the causative micro-organism runs a course parallel to the responses to the other microbial antigens. It was not possible, from these lymphocyte transformation studies, to obtain evidence for a specific boost of the response to the causative micro-organism during the convalescense period.

During the recovery phase of the transformation response, evidence for a shift in antigen optimum

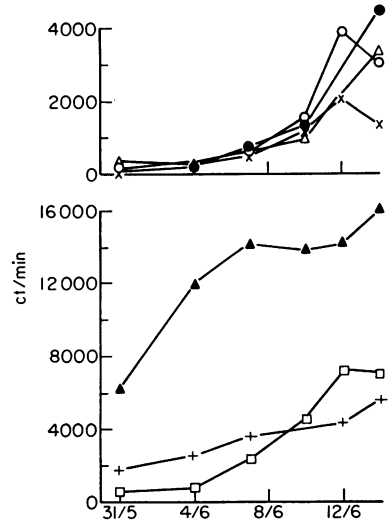

FIG. 1. Lymphocyte transformation studies in a patient (H.G.) with uncomplicated meningococcal meningitis. Thymidine incorporation in response to mitogens (b) and to microbial antigens (a). (●) *C. albicans*; (△) PPD; (○) *N. meningitidis*; (×) *E. coli*; (▲) PHA; (□) Con A; (+) PWM.

in vitro, towards higher sensitivity of the lymphocytes to the causative micro-organism, was found in three out of eight cases. This shift was of minor degree, the optimum antigen concentration being four- to sixteen-fold lower after approximately 2 weeks of illness than at the onset (Fig. 3). In cultures containing supraoptimal numbers of bacteria, the response to an added dose of PHA was depressed, demonstrating that such high concentrations of bacteria are toxic to lymphocytes.

One year after the illness, all lymphocyte transformation responses were within the range found in healthy persons by our laboratory; the responses to the causative micro-organism were similar to those obtained when the patient was discharged.

Thymidine incorporation in unstimulated cultures was initially low (average value in cultures of 72 hr 95 ct/min, of 120 hr 130 ct/min). Maximal unstimulated values were obtained between days 4 and 9 (average 293 ct/min and 247 ct/min respectively), followed by a decline in four out of eight patients (average values in cultures set up before discharge 157 ct/min and 236 ct/min respectively).

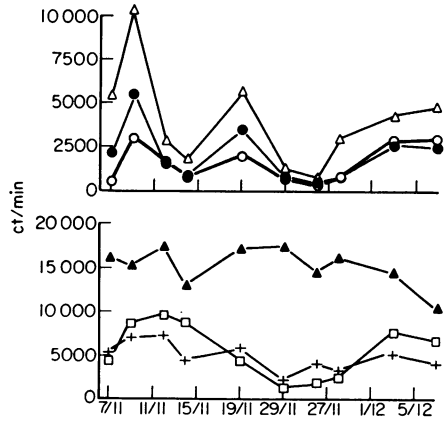

FIG. 2. Lymphocyte transformation studies in a patient (T.L.) with meningococcal meningitis complicated with arthritis on days 7–21. Thymidine incorporation in response to mitogens (b) and to microbial antigens (a). (△) PPD; (○) *N. meningitidis*; (●) *C. albicans*; (▲) PHA; (□) Con A; (+) PWM.

TABLE 1. Lymphocyte transformation studies in eight patients with bacterial meningitis. Thymidine incorporation by cells from the initial blood sample and after recovery. If a secondary depression of lymphocyte transformation occurred, the values during relapse and recovery are given in parentheses. Duration of cultures with mitogens 72 hr, with antigens 120 hr. As unrelated microbial preparations were employed PPD and an extract of *C. albicans* which gave essentially similar results

Patient	Causative micro-organism	No addition		Phytohaemagglutinin		Con A		Pokeweed mitogen	
		Initial ct/min	Recovery ct/min	Initial ct/min	Recovery ct/min	Initial ct/min	Recovery ct/min	Initial ct/min	Recovery ct/min
H.G.	*N. meningitidis*	30	80	6200	14,200	640	7300	1810	4500
K.S.	*N. meningitidis*	150	480	7000	17,900	5500	11,800	5800	10,300
E.L.	*N. meningitidis*	150	110	12,400	11,900	n.d.	n.d.	n.d.	n.d.
K.H.	*N. meningitidis*	20	160	5600	7100	n.d.	n.d.	n.d.	n.d.
T.L.	*N. meningitidis*	50	290	16,300	17,700	4800	9800	5600	7200
		(130)	(200)	(14,600)	(16,500)	(1330)	(7800)	(2200)	(5300)
M.N.	*S. pneumoniae*	110	180	6700	11,600	n.d.	4200	n.d.	2700
		(360)	(300)	(8500)	(11,900)	(1700)	(3500)	(1500)	(3100)
J.C.	*S. pneumoniae*	160	280	1630	9700	440	680	320	1430
		(360)	(270)	(6200)	(18,200)	(480)	(4900)	(1010)	(2500)
B.S.	*E. coli*	110	310	6100	7800	420	2200	1880	3100

No addition		Causative micro-organism		Unrelated micro-organism	
Initial ct/min	Recovery ct/min	Initial ct/min	Recovery ct/min	Initial ct/min	Recovery ct/min
70	190	190	3900	360	3300
180	330	2800	4700	2300	4300
140	230	460	1690	280	1460
60	110	170	590	n.d.	n.d.
90	320	540	3000	2100	5500
(120)	(190)	(410)	(3000)	(490)	(2800)
130	260	340	800	380	760
(360)	(240)	(380)	(860)	(740)	(1240)
170	160	160	430	150	1090
(150)	(510)	(280)	(2300)	(510)	(9600)
210	200	740	2300	620	2700

n.d. = Not done.

Blood lymphocytes and monocytes

The lymphocyte concentration in the blood was below normal in the acute phase of the illness (average concentration 990/μl) and recovered concomitantly with the clinical recovery of the patient (average at discharge 2100/μl). The initial average monocyte concentration was 340/μl; the concentration increased to elevated values during early convalescense (average maximum 950/μl) and then again decreased (average at discharge 390/μl). Correction for the number of monocytes present in the transformation cultures, by expressing the thymidine incorporation per 10^5 lymphocytes originally cultured, does not affect the conclusions to be drawn.

Antibody studies

The level of serum antibodies against *N. meningitidis*, quantified by the complement fixation test, rose to near maximum within the first 8 days of the illness (Fig. 4); higher titres were observed when the causative strain was employed as antigen than when the standard meningococcal preparation containing

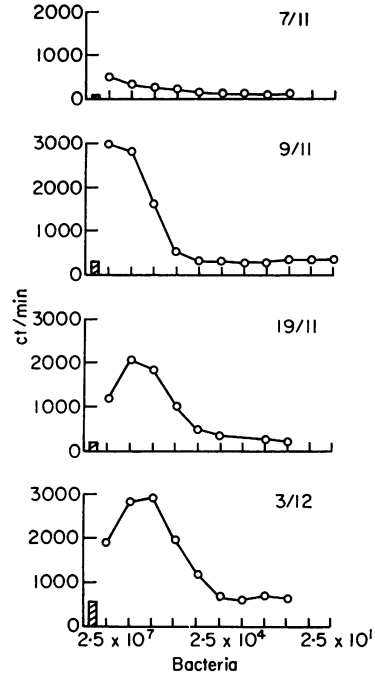

FIG. 3. Dose-response curves for lymphocyte transformation in response to heat-killed meningococci on 4 different days during the illness and convalescence of a patient with meningococcal meningitis. Abscissa: number of heat-killed bacteria per culture of 10^5 mononuclear cells. Ordinate: thymidine incorporation. Columns: results in unstimulated cultures.

ten strains was used. In uncomplicated cases (Fig. 4b), the titre decreased rapidly, whereas in patients with a complicated course (Fig. 4a) the antibody response was more sustained.

The number of precipitating antibodies against *N. meningitidis* demonstrable in the patients' sera showed a rise over the first 11 days on the average and thereafter declined (Fig. 5). At maximum, the mean number of precipitating antibodies demonstrated was 3, the largest number observed was 7.

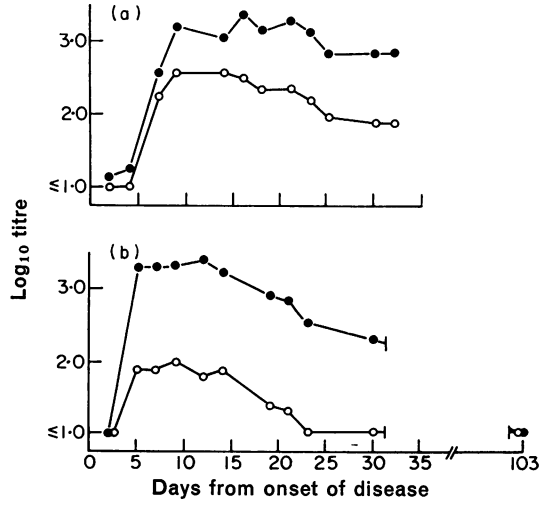

FIG. 4. Results of the meningococcal complement fixation test on serum from patients with meningococcal meningitis: (●) with the patient's own meningococcus as antigen; (○) with the standard meningococcal preparation. (a) Patient with arthritis on days 7–21; (b) patient with uncomplicated course.

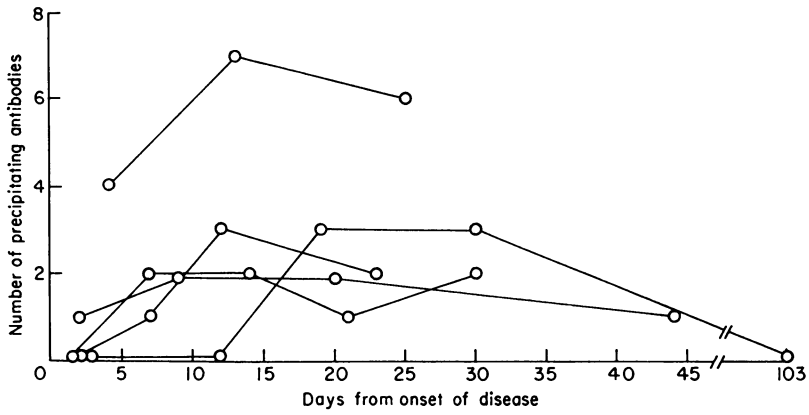

FIG. 5. Number of precipitating antibodies against *N. meningitidis* in the five patients with meningococcal meningitis.

Both patients with pneumococcal meningitis had one precipitating antibody against pneumococcal antigens in their first serum sample; a rise was observed to two and three precipitating antibodies respectively. The one patient with coli-meningitis had six precipitating antibodies against *E. coli* antigens in the first serum sample; the number decreased to 4 on day 10 and then again increased to 6 on day 30.

Serum proteins

The changes in concentration of the serum proteins studied are shown in Fig. 6. The concentrations of albumin were low initially and rose progressively towards normal values. All acute-phase reactants

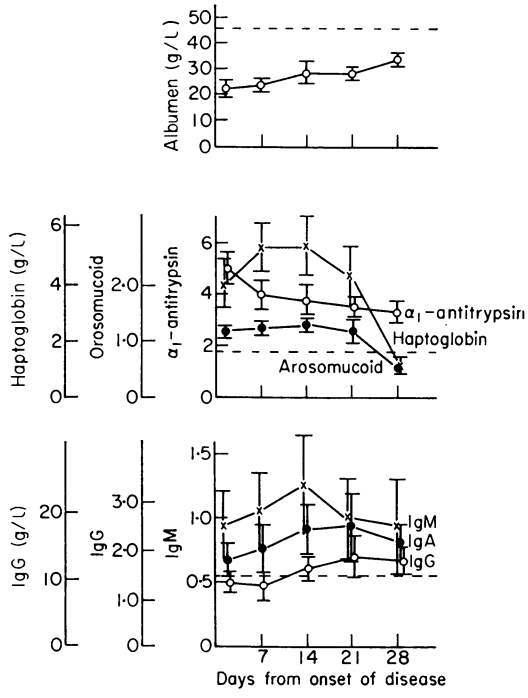

FIG. 6. Changes in the concentrations of selected serum proteins in seven patients with acute bacterial meningitis (the patient with a protracted course and several relapses is excluded). Average values ±s.e.m. (- - -) mean concentration in normal adults.

studied were elevated at first sampling; α_1-antitrypsin showed no further rise in seven out of eight patients, whereas the haptoglobin concentration rose in six out of eight patients with a maximum at 1–2 weeks after the onset of illness. Haptoglobin and orosomucoid concentrations were down to normal 1 month after the acute infection.

Among the immunoglobulins studied, IgM showed a rise in six out of eight patients, with maximum 1–2 weeks after the onset of illness. IgA and IgG rose in seven out of eight patients; the rise was more prolonged, with maximum at 3 weeks.

DISCUSSION

In these patients with acute and severe infection, rises in serum antibodies were demonstrable on day 4 of illness. In the patients with meningococcal meningitis, the maximal titre of complement-fixing antibodies was reached around day 8; in crossed immunoelectrophoresis, the number of precipitating antibodies showed a maximum around day 10. It has been shown (Hoffmann & Edwards, 1972) that the levels of bactericidal antibody and of haemagglutinin antibody in meningococcal infection rise even faster than the concentration of complement-fixing antibody. The IgM concentration in serum showed a maximum 1–2 weeks after the onset of illness, whereas IgG and IgA continued to rise until 3 weeks after the first symptoms of the infection. It is thus apparent that the concentration of specific antibodies peaks earlier than the total immunoglobulin concentration. Several explanations of this discrepancy are possible. It is compatible with the concept of the immune system as a self-regulatory network in which responses directed against the idiotypes of antibodies produced against an extraneous challenge limit the immune response (Jerne, 1973, Hoffmann, 1975), implying that the latter part of the increase in total Ig reflects this regulatory function of Ig production. Besides, elevated titres of antiglobulin antibodies (anti-Fab IgG and anti-Fc IgG) have been demonstrated in association with infection in man (Waller, Miller & Kelly, 1975).

The *in vitro* lymphocyte-transformation responses were depressed during the acute phases of illness. The transformation induced by the causative micro-organism was not different from this general pattern, and the response to the causative micro-organism was not higher after clinical cure than in healthy controls studied in our laboratory. Thus, non-specific factors seem to be the major determinant of the lymphocyte transformation response to washed and heat-killed bacteria as employed in the present study; it will be important to investigate whether other methods of antigen preparation for *in vitro* lymphocyte stimulation will allow a more specific response to be detected. Our results indicate that whenever transformation responses to microbial antigens are studied, preparations of other micro-organisms should be included as controls; otherwise, the sequence shown in Fig. 1 for *N. meningitidis* might easily be mistaken for the development of a specific immune response.

One mechanism behind this apparent non-specificity may be the cross-reactions between antigens from a wide variety of bacteria that have been demonstrated by crossed immunoelectrophoretic analyses (Høiby, 1975; Høiby, Hertz & Andersen, 1976). Another possibility is that mitogenic substances present in the bacteria determine the lymphocyte response; mitogenicity has been demonstrated in many bacterial preparations (Peavy *et al.*, 1973; Taranta, 1974; Forsgren, Svedjelund & Wigzell, 1976; Andersen *et al.*, 1976).

A shift in the concentration of antigen inducing maximal lymphocyte transformation was carefully looked for during the period of evolution of the humoral response. Such a shift was found in only three out of eight patients; the magnitude of the shift was small (Fig. 3) and not comparable to the increases in avidity of antibodies observed during immunization (Siskind & Benacerraf, 1969). A similar small shift has previously been observed in man during immunization with *Brucella abortus* (Andersen, Søborg & Sørensen, 1971). The mechanisms involved are not fully elucidated; a possible factor may be the production in the culture of specific antibodies, with the formation of antigen–antibody complexes which are more stimulatory than antigen itself (Oppenheim *et al.*, 1973). However, it must be remembered that the number of antigen-sensitive cells present in the culture influences the profile of dose-response curves for lymphocyte transformation (Rubin & Wigzell, 1974).

The depression of lymphocyte transformation was related to clinical parameters of disease activity. In uncomplicated cases with rapid recovery, depression was most marked in the first sample (Fig. 1); in these cases bacterial antigen, if found in the blood, was present only in the first sample (Hansen *et al.*, 1976). In patients with complications, depression of lymphocyte transformation was observed in periods with fever and other signs of disease activity (Fig. 2), and in the patient with relapsing pneumococcal meningitis, in whom bacterial antigen was repeatedly found in the blood during relapses, depression of lymphocyte transformation followed the same pattern.

The lymphocytopenia occurring in the blood during the acute phase of illness does not explain the decreased transformation response, since the number of cells per culture was kept constant. Little is known about lymphocyte kinetics in acute infection, but shifts of active lymphocyte populations from the blood to the tissues can not explain all of our findings: During acute bacterial infections normal (Aiuti *et al.*, 1973) or slightly decreased (Niklasson & Williams, 1974) proportions of T lymphocytes have been found in the blood; since normal T lymphocytes are activated by PHA, the marked depression of the PHA response observed in several of our patients indicates a depression of lymphocyte function. The treatment given does not explain the depression of lymphocyte transformation, since similar changes were seen during complications later in the course, when no change in therapy was initiated. The transformation studies were performed in 20% pooled normal human serum, after washing of the cells. Therefore, the results indicate that the depression of lymphocyte transformation resides in the cells themselves or in factors tightly bound to their surface. One practical consequence is that studies of lymphocyte transformation, in order to investigate possible primary immune deficiency, must be interpreted with special caution if carried out during or shortly after bacterial infection.

The mechanisms causing this non-specific depression of lymphocyte function are not known but are amenable to further experimentation which should include phagocyte dysfunction and blocking factors from plasma adsorbed to the cells. A possible parallel *in vivo* is the temporary suppression of i.c. reactions of delayed type which is best known in acute viral illness but has also been described in acute bacterial infection (Kantor, 1975). It will be of interest to investigate whether the depression of lymphocyte function demonstrated *in vitro* in the present study is causally related to the eruption of herpes labialis which is seen in the majority of adult patients with bacterial meningitis (Jessen, 1976 in preparation).

The able technical assistance of Vita Weibull and Jette Hassing is gratefully acknowledged. We thank Bent Rubin for helpful discussions during the preparation of the manuscript. This work was supported by the Danish Medical Research Council and by the Daell Foundation.

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