Clin. exp. Immunol. (1977) **30**, 44–49.

Blastogenic response of human lymphocytes to human cytomegalovirus

J. L. WANER & JOAN E. BUDNICK *Department of Tropical Public Health, Harvard School of Public Health, Boston, Massachusetts, U.S.A.*

(*Received* 18 *January* 1977)

SUMMARY

A method was developed for measuring the blastogenic response of human lymphocytes to human cytomegalovirus (CMV). Viral and control antigens were prepared by extracting disrupted infected and uninfected cell cultures with an alkaline buffer. Lymphocytes from ten donors with complement-fixing (CF) antibody exhibited a blastogenic response, whereas cells from ten seronegative donors did not. A relationship between the stimulation index (SI) and the results of neutralization (NT), indirect haemagglutination (IHA) or CF tests was not observed. The maximum blastogenic response occurred after 5 to 7 days of incubation and was usually greater when the cultures were supplemented with homologous plasma instead of sera. The presence of CMV antibody in the supplementary sera did not appear to affect the reactivity of the lymphocytes.

INTRODUCTION

The role of cell-mediated immunity (CMI) in cytomegalovirus (CMV) infection in man is poorly understood. Transformation of lymphocytes in response to specific CMV antigenic stimulation is one correlate of CMI which remains uncharacterized. To assess the cellular immune status of patients with CMV disease a standardized test for lymphocyte transformation is required, as is baseline information regarding CMI competence in asymptomatically infected individuals. It was reported (Thurman *et al.*, 1973) that CMV-infected cells elicited a blastogenic response with lymphocytes of two CMV-infected patients but not with those from a seropositive, but asymptomatic control patient. In contrast to the results on exposure to infected cells, lymphocytes did not transform when cultured with purified CMV, suggesting that cell-associated viral antigen was needed to stimulate lymphocyte blastogenesis.

We now report that a preparation of CMV antigens, also useful in two tests for humoral immunity, induces significant transformation of lymphocytes from asymptomatic seropositive individuals. The dynamics of the lymphocyte transformation test are described. The reactivity of lymphocytes from asymptomatic donors is described and correlated with levels of CMV antibody.

MATERIALS AND METHODS

Cell cultures. Primary human fibroblast cultures were prepared from foetal lung tissue obtained locally. Cell cultures were grown at 36°C and maintained in minimum essential medium (MEM) supplemented with 5% foetal calf serum, 100 u/ml of penicillin and 100 μg/ml of streptomycin.

Virus. Cell cultures infected with the AD169 (passages 79–83) strain of CMV were the source of antigenic material. The preparation of cell-free infectious stocks has been described previously (Waner & Budnick, 1973).

Preparation of antigen. CMV and control antigens were prepared from infected and uninfected cell cultures, respectively, by the glycine-buffered saline (GBS) extraction procedure described previously, except that the pH of the buffer used was 8·0 instead of 9·0 (Waner, Weller & Stewart, 1976).

Correspondence: Dr J. L. Waner, Department of Tropical Public Health, Harvard School of Public Health, 665 Huntington Avenue, Boston, Massachusetts, U.S.A.

Serological tests. The methods for the complement-fixation (CF), indirect haemagglutination (IHA) and neutralization tests have been described (Waner *et al.*, 1976). The control and CMV antigen preparations used in the assays of lymphocyte blastogenesis also served as antigens in the CF and IHA tests. Infections stocks of strain AD169 were used in the neutralization tests.

Collection of lymphocytes and sera. Venous blood was obtained from eleven male and nine female donors ranging in age from 21 to 62 years, and 50 μg/ml of preservative-free heparin was added to each portion. The heparinized blood was layered on an equal volume of a sterilized Ficoll–Hypaque gradient prepared by mixing 2·4 parts of 9% Ficoll (w/v) with 1 part of 34% Hypaque. After centrifugation of the gradient with blood at ambient temperatures for 50 min at 500 *g*, the cells which banded at the interface were collected with a pipette and washed once with medium. The remainder of the blood from each donor was allowed to clot overnight at 4°C. Serum was then removed after centrifugation at 250 *g* for 10 min, inactivated at 56°C for 30 min and stored at −20°C.

Lymphocyte cultures. Cellular viability was determined by the dye exclusion method using 0·2% trypan blue. Lymphocyte cultures were prepared in 17× 100 mm plastic tubes by suspending 10^6 viable cells in 0·8 ml of medium 199 supplemented with 100 u/ml of penicillin, 100 μg/ml of streptomycin and 180 mg/100 ml of sodium bicarbonate. Medium 199 was also used as diluent for the antigens and phytohaemagglutinin (PHA). PHA was purchased from Wellcome Reagents Limited, Beckenham, England. Each culture received 0·1 ml of either homologous serum or plasma (obtained from the top of the gradient) and 0·1 ml of PHA (0·5 μg), of control antigen, of CMV antigen or of medium. Cultures were incubated at 37°C in a 5% CO_2 atmosphere, usually for 7 days, a period established as the optimum time for assaying lymphocytic blastogenesis as described below. Then 1 μCi of [^{3}H]thymidine (New England Nuclear) was added to each culture and 18 hr later the cultures were centrifuged at 500 *g* for 20 min at ambient temperature. Supernatants were discarded, the tubes placed in an ice bath and the cell pellets resuspended in 1 ml of cold saline; 0·05 ml of 5 N perchloric acid (PCA) and 4·5 ml of 0·2 N PCA were added sequentially to each tube to obtain the acid-precipitable fraction. Each sample was filtered through a 2·4 cm Whatman GF/C filter paper disc; each disc was placed in 10 ml of Scintiverse (Fisher Scientific) scintillation fluid and counted in a Searle Mark III scintillation counter. Lymphocyte cultures were prepared in duplicate or triplicate and mean counts determined. Reactions were considered positive if the incorporation of [^{3}H]thymidine by the stimulated cultures were at least twice that of the comparable control cultures. The stimulation index (SI) was calculated by dividing the number of ct/min incorporated into CMV antigen, or PHA-treated cultures, by the number of ct/min incorporated into control antigen, or medium-treated cultures, respectively. Cultures incubated only with medium provided the baseline incorporation values.

RESULTS

Determination of the optimum antigen dilution for lymphocyte blastogenesis

The optimum dilution of CMV antigen was determined for each batch of antigen by testing two-fold dilutions of CMV and of control antigens in cultures of lymphocytes obtained from a donor known to react with CMV antigen. A representative determination is shown in Table 1. When the optimum

TABLE 1. Determination of the optimum antigen dilution for lymphocyte transformation

CMV and control antigen dilutions	Uptake of [^{3}H]thymidine (ct/min)		
	Control antigen	CMV antigen	SI
1:2	5391±240	39022±1681	7·23
1:4	4225±325	58232±1210	13·78
1:8	4333±216	8227±202	1·89
Baseline*	4632±580	—	—
PHA	127991±9050	—	—

* Cells incubated only with medium.

dilution of an antigen preparation was found to be 1:4, the CMV and control antigen stocks were diluted 1:4 in subsequent assays. The optimum dilutions of three stocks of antigen prepared in this laboratory have ranged between 1:4 and 1:8. Undiluted antigen and undiluted glycine-buffered saline, used as a control in place of the antigen, were slightly toxic to lymphocyte cultures; the use of two-fold dilutions in the medium eliminated toxicity.

TABLE 2. Determination of the optimum time for assaying the reactivity of lymphocytes cultured with CMV antigen

Donor	Days incubated	Baseline*	PHA	SI (PHA)	Control antigen	CMV antigen	SI (CMV)
J.C.	3	1874±48	236989±8790	126·46	1663±951	13660±955	8·21
(Seropositive)	5	3172±183	218657±8430	68·93	4015±372	76314±5680	19·01
	7	1931±58	62735±4040	32·48	4858±411	96051±8140	19·76
	9	1382±98	42897±3104	31·03	2835±108	35961±1151	12·68
J.W.	3	1195±46	93872±7880	78·55	1415±152	3039±210	2·15
(Seropositive)	5	1806±89	153058±8021	84·74	3887±183	30156±2834	7·76
	7	1755±33	69955±4150	39·86	7104±305	79758±4070	11.23
	9	1110±41	36634±3780	33·00	22272±991	52239±4320	2·35
J.B.	3	1320±110	114398±5255		5672±450	4335±256	
(Seronegative)	7	1064±44	66496±3820		9968±384	7744±573	
E.A.	3	2263±185	389926±15075		2228±286	1628±184	
(Seronegative)	7	2465±236	291608±10843		3001±253	3340±254	

* Cells incubated only with medium.

Determination of the optimum time for assaying the reactivity of lymphocytes cultured with CMV antigen

Cultures of lymphocytes from the CMV-reactive donors were assayed at 3, 5, 7 and 9 days after the addition of antigens or PHA. The maximum stimulation indexes were obtained after 5–7 days of incubation of lymphocytes with antigen (Table 2). The maximum response to PHA, however, occurred between 3 and 5 days. In assays performed with lymphocytes from three other donors, the maximum PHA response occurred at 3 days. The baseline or background levels of [^{3}H]thymidine incorporation were relatively constant throughout the time of incubation. Lymphocytes from certain donors consistently exhibited higher background values at all time intervals. Lymphocytes incubated with control antigen, however, often demonstrated increased reactivity with time. Subsequent assays of lymphocyte blastogenesis were done at 7 days. The cultures incubated with PHA were assayed at the same time as the cultures incubated with antigen as a matter of convenience, since the PHA response was used only as a non-specific indicator of the capacity of the lymphocytes to undergo blastogenesis.

The effect of sera and plasma on the specific blastogenic reaction of lymphocytes

Lymphocyte cultures from eight donors, whose lymphocytes were known to respond to CMV antigen, were supplemented with either homologous serum, plasma, inactivated plasma (56°C for 30 min) or a heterologous serum. Lymphocytes from one donor were cultured with the four different supplements separately and assayed for blastogenesis to CMV antigen in a single experiment. The SI of lymphocytes from four of the eight donors were highest in cultures supplemented with inactivated plasma; two were most reactive with unheated plasma (Table 3). Two of the eight showed maximum reactivity with supplementary serum in the lymphocyte cultures. The background incorporation varied considerably between members of each group and was generally greatest in cultures supplemented with homologous plasma and lowest in cultures supplemented with heterologous sera.

The effect of antibodies to CMV on the blastogenic response

Lymphocytes were obtained from donors with CF antibodies to CMV and cultured in the presence of homologous serum or of heterologous serum possessing approximately the same antibody titre as the donor's serum, or with heterologous serum free of antibody. Although the effect of antibody on the blastogenic response cannot be definitely evaluated, it is clear that the presence of antibody in the culture system did not inhibit blastogenesis of lymphocytes from reactive donors (Table 4).

TABLE 3. The effect of incorporation of sera or plasma on the blastogenic response of lymphocytes from CMV-reactive donors

	Donor serum		Donor plasma		Donor plasma (inactivated)		Heterologous serum	
Donor	SI	Baseline*	SI	Baseline	SI	Baseline	SI	Baseline
E.M.	13·66	6352±981	12·76	7284±453	24·69	5353±101	7·82	4290±125
G.K.	10·46	3396±208	1·29	7967±640	14·60	2184±93	12·93	992±53
J.C.	9·61	3085±264	32·63	4459±386	18·39	9341±261	13·78	5932±215
M.T.	8·42	2689±463	5·81	10505±937	23·74	2844±222	8·24	3741±404
S.P.	6·76	6360±522	3·65	7641±20	32·10	1879±95	5·82	4728±380
L.K.	6·74	1491±48	2·82	3649±240	5·60	3344±155	3·82	1462±86
T.W.	5·30	13215±852	8·91	13841±671	9·41	8308±450	14·66	2603±147
J.W.	4·45	5392±280	11·30	7723±575	6·93	4002±191	9·66	2430±83
Average	8·17	5247	9·89	7883	16·93	4656	9·02	3792

* Cells incubated only with medium.

Comparison of the lymphocyte response of CMV-reactive donors with donor serological status

Lymphocytes from ten of the twenty blood donors studied reacted with CMV antigen as evidenced by [^{3}H]thymidine incorporation. All ten reactors possessed CF antibody in their sera. CMV CF, IHA or neutralizing antibody could not be detected in the sera from the ten donors whose lymphocytes were unreactive to CMV antigen (Table 5). One of the ten donors with reactive lymphocytes did not have detectable IHA or neutralizing antibodies but had CF antibody. Table 5 compares the maximum SI obtained from the ten reactors with the serological results obtained with sera taken from the same blood specimen which provided the lymphocytes. A direct relationship between the SI and the results of individual serological tests was not observed, nor were any direct relationships observed when the SIs obtained in experiments employing homologous sera heterologous sera or plasma (whether inactivated or not) were compared to the serological results.

TABLE 4. The effect of antibodies to CMV on the blastogenic response of lymphocytes in culture

		Lymphocyte culture supplement		
		Donor serum	Heterologous sera	
Donor	Antigen		Ab-positive	Ab-negative
E.M. (128)*	Control Ag	6736±324	15082±1873	8639±1158
	CMV Ag	92025±4791 (13·66)†	68805±460 (4·56)†	67576±2095 (7·82)†
K.M. (64)	Control Ag	10260±211	7820±286	14607±631
	CMV Ag	50335±1820 (4·90)	48539±1503 (6·18)	22904±851 (1·57)
S.P. (64)	Control Ag	8575±468	3777±138	8994±789
	CMV Ag	57972±2420 (6·76)	50238±2236 (13·30)	52362±1715 (5·82)
N.R. (64)	Control Ag	2591±136	9912±1054	6938±985
	CMV Ag	40030±1289 (16·61)	50343±1399 (5·08)	30969±585 (4·47)

* CF antibody titre.

† Numbers in parentheses show SI.

TABLE 5. Comparison of the lymphocyte response of CMV-reactive donors with their serological status

	Lymphocyte response to control and CMV Ag			Serological tests			
						Neutralization†	
Donor	Baseline*	Control Ag	CMV Ag	CF	IHA	C′ positive	C′ negative
R.C.	4459±386	5559±399	181409±6761 (32·63)‡	128	512	64	32
S.P.	1879±95	1980±154	65563±4381 (32·10)	64	128	64	32
E.M.	5353±101	5818±490	143641±10555 (24·69)	128	8	32	16
M.T.	2844±222	4886±206	116032±10050 (23·74)	32	256	16	8
N.R.	1892±104	2591±136	40040±1289 (16·61)	64	256	64	32
T.W.	2603±147	2377±16	34845±768 (14·66)	32	128	16	<8
G.K.	2184±93	1622±159	23676±1114 (14·60)	64	128	32	16
J.W.	7723±575	5172±455	58490±2026 (11·30)	32	64	16	8
L.K.	1491±48	1579±110	10643±680 (6·74)	16	0	<8	<8
K.M.	1582±83	7820±286	48539±1503 (6·18)	64	64	16	8

* Cells incubated only with medium.
† C′ positive, complement in test; C′ negative, complement not in test.
‡ Numbers in parentheses show SI.

DISCUSSION

Other investigators have reported a requirement for antigen associated with intact CMV-infected cells to induce blastogenesis in lymphocytes from symptomatic patients; lymphocytes from asymptomatic donors were not reactive (Thurman *et al.*, 1973). Intact, CMV-infected cells present only viral-induced surface antigens to the lymphocytes. In our study, the successful induction of blastogenesis in lymphocytes from asymptomatic donors may reflect the use of a greater antigenic concentration or different antigens. Extraction of CMV-infected cells with GBS results in a preparation containing soluble antigen (Waner, 1975) and antigens associated with small pieces of membranous host material derived from surface and interior cell structures (Waner, unpublished observation). Larger quantities of antigen of a more diverse nature are thus available to the CMV-sensitive lymphocytes present in cultures prepared from asymptomatic donors.

Several procedural advantages, of importance to the clinical laboratory, are derived from the use of GBS CMV antigen. Antigen extracted from infected cells allows the use of a control antigen prepared from uninfected cell cultures and thus increases the accuracy of the calculated SI. Since infectious virions are not present in GBS-extracted antigen, the possible role of CMV infection of the lymphocytes is eliminated (Pagano, 1975). The CMV and control antigens may also be used in CF and IHA tests.

The same antigen preparation was used to induce lymphocyte transformation and in the performance of the CF and IHA tests. There was no correlation between the SIs and the antibody titres of the donors. Fluctuations in lymphocyte reactivity are expected and fluctuations in CMV CF antibody levels have also

been reported (Waner, Weller & Kevy, 1973). The independent reactivity of the CMI and humoral systems to different CMV antigens may be an additional factor. The host–CMV relationship is in a state of dynamic equilibrium. Latent infections that are reactivated would result in antigens that stimulate immune mechanisms to suppress the exacerbation, while the ubiquitous nature of CMV must result in periodic primary exposures to antigens of different strains. How, and in what order of events, the immune system deals with these challenges is not clear, but at any given time, antibody titres could be high to one antigen while lymphocyte reactivity is low or vice versa. If more than one strain of CMV had been used as a source of antigen in this study, the observed patterns of lymphocytic and serological reactivities might have been different.

Clinical data have shown that susceptibility to CMV infection increases in immunosuppressed patients, although humoral immunity may be unimpaired (Anderson & Spencer, 1969; Craighead, Hanshaw & Carpenter, 1967; Duvall *et al.*, 1966; Henson *et al.*, 1972). The presence of antibody may be an indication of exposure to CMV but assessment of the host's immune competence to CMV might be best accomplished with specific CMV antigens known to react in tests of CMI.

The authors thank Dr Thomas H. Weller for reviewing the manuscript. This work was supported by grant AI-01023 from the U.S. Public Health Service.

REFERENCES

ANDERSON, H.K. & SPENCER, E.S. (1969) Cytomegalovirus infection among renal allograft recipients. *Acta med. scand.* **186**, 7.

CRAIGHEAD, J.E., HANSHAW, J.B. & CARPENTER, C.B. (1967) Cytomegalovirus infection after renal allotransplantation. *J. Am. med. Ass.* **201**, 725.

DUVALL, C.P., CASAZZA, A.R., GRIMLEY, P.M., CARBONE, P.P. & ROWE, W.P. (1966) Recovery of cytomegalovirus from adults with neoplastic disease. *Ann. int. Med.* **64**, 531.

HENSON, D., SIEGEL, S.E., FUCCILLO, D.A., MATTHEW, E. & LEVINE, A.S. (1972) Cytomegalovirus infections during acute childhood leukemia. *J. infect. Dis.* **126**, 469.

PAGANO, J.S. (1975) Diseases and mechanisms of persistent DNA virus infection: latency and cellular transformation. *J. infect. Dis.* **132**, 209.

THURMAN, G.B., AHMED, A., STRONG, D.M., KNUDSEN, R.C., GRACE, W.R. & SELL, K.W. (1973) Lymphocyte activation in subacute sclerosing panencephalitis virus and cytomegalovirus infections. *J. exp. Med.* **138**, 839.

WANER, J.L. (1975) Partial characterization of a soluble antigen preparation from cells infected with human cytomegalovirus: properties of antisera prepared to the antigen. *J. Immunol.* **114**, 1454.

WANER, J.L. & BUDNICK, J.E. (1973) Three day assay for human cytomegalovirus applicable to serum neutralization tests. *Appl. Microbiol.* **25**, 37.

WANER, J.L., WELLER, T.H. & KEVY, S.V. (1973) Patterns of cytomegaloviral complement-fixing antibody activity: a longitudinal study of blood donors. *J. infect. Dis.* **127**, 538.

WANER, J.L., WELLER, T.H. & STEWART, J.A. (1976) Cytomegalovirus. *Manual of Clinical Immunology* (ed. N. R. Rose and H. Friedman), p. 423. American Society for Microbiology, Washington D.C., U.S.A.