Brief Definitive Report

B CELL ACTIVATION BY CYTOMEGALOVIRUS*

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Infectious mononucleosis (IM) is a lymphoproliferative disorder accompanied by a transient acquired immunodeficiency (1). It is characterized by an atypical lymphocytosis that is principally of T cell origin. Epstein-Barr virus (EBV) is the major cause of the disease and the pathogenesis of this infection has been well studied. The T cell lymphocytosis is generally thought to be a response to EBV-infected and -activated B cells. It includes cytotoxic cells and suppressor cells capable of inhibiting B cell outgrowth and immunoglobulin (Ig) synthesis (2).

In striking contrast, very little is known about how cytomegalovirus (CMV), the second leading cause of the disease, generates similar activated T cell responses (3) and reversals in normal helper/suppressor T cell ratios (4). All current available evidence indicates that human CMV does not replicate in normal B lymphocytes (1). However, some direct or indirect nonspecific polyclonal interaction with these cells has been implied. Patients with CMV mononucleosis, even more than their EBV-infected counterparts, have unusually high frequencies of circulating Ig-secreting lymphocytes (5), as well as increases in antibodies that are apparently irrelevant to viral antigens (6, 7). In this paper we present evidence that CMV is capable of inducing nonspecific polyclonal Ig synthesis and suggest that this may explain some of the serologic abnormalities of CMV infections. The finding is of particular interest in view of the fact that hypergammaglobulinemia has been reported in CMV-infected patients with the acquired immunodeficiency syndrome (AIDS) and that in vivo polyclonal activation of B cells, possibly by virus, has been suggested as contributory to this facet of the disease (8).

Materials and Methods

Cell Preparation and Culture. Heparinized peripheral blood was separated by flotation on Lymphocyte Separation Medium (LSM; Litton Bionettics, Charleston, SC). T cells were depleted by rosetting with sheep erythrocytes (E; reference 9) and flotation on LSM. In some experiments this procedure was repeated to reduce T cell contamination to <1%. Leukocytes were differentiated by the ability to form E-rosettes or stain for nonspecific esterases (10). Cells with neither of these properties were designated "B cells." For plaque assays, 2×10^6 cells were incubated in 12-mm × 75-mm plastic tissue culture tubes with virus- or mock-infected control preparations in a total volume of 1.2 ml RPMI 1640 (Gibco Laboratories; Grand Island, NY) containing 10% heat-inactivated fetal calf serum (FCS). For measurement of Ig synthesis, 2×10^5 cells were incubated in a total volume of

* Supported in part by grant CA33654 from the U. S. Public Health Service.

100 μl in 96-well round bottomed tissue culture plates with virus, control material, or pokeweed mitogen (PWM; Gibco). After 6 d in culture, 100 μl media were added to each well. On days 7, 9, 12, and 14, 100 μl supernatant media were exchanged for fresh virus-free media and the Ig contents of the removed supernatants were measured.

Virus. EBV was obtained from spent culture media of the MCUV5 marmoset line (a gift of Dr. George Miller, Yale University) which had been clarified by low speed centrifugation. The virus was pelleted by centrifugation at 20,000 *g* for 90 min, resuspended in 1/250 original volume of RPMI and filtered through a 0.45-μm filter.

Towne strain CMV, grown in WI_{38} cells, was kindly donated by Dr. E.-S. Huang (University of North Carolina) and passaged at low multiplicity of infection (m.o.i.) in mycoplasma-free human foreskin fibroblasts (HFF). Infected culture medium was centrifuged for 10 min at 600 *g* to remove cell debris. Control material was prepared by passaging mock-infected WI_{38} cells in HFF. A fresh isolate of CMV was obtained from the Shands Hospital Virology Laboratory (Gainesville, FL) and passaged six times in HFF.

CMV infectivity was determined by counting plaques 10 d after infection of monolayers of HFF grown in 24-well tissue culture plates. Inactivated virus was prepared by irradiation of 1 ml of virus, for 30 min, in a 60-mm diameter petri dish at 20 cm from a short-wave germicidal lamp (GTE Sylvania Inc.), after which, the virus induced no cytopathic effect in HFF maintained for 3 wk post infection.

Plaque Assay. Ig-secreting cells were enumerated in a reverse hemolytic plaque assay (11). Leukocytes were mixed with guinea pig serum (preadsorbed with sheep erythrocytes coupled to Staphylococcal Protein A [Protein A; Pharmacia, Piscataway, NJ]) and with sheep erythrocytes coupled to rabbit anti-human immunoglobulins (IgM,IgG,IgA; Cappel Laboratories, Cochranville, PA). The mixtures were incubated in Cunningham chambers for 8–10 h and plaques counted microscopically. Results were expressed as plaque-forming cells (PFC)/10^6 viable cells.

Immunoglobulin Assays. Immunoglobulins in culture supernatants were measured by a double sandwich micro-ELISA method (12), using appropriate concentrations of rabbit anti-human immunoglobulins (IgM,IgG,IgA), anti-human IgM, peroxidase-conjugated rabbit anti-human Ig (Cappel) and the substrate hydrogen peroxide with 5-amino salicylic acid (Sigma Chemical Co., St. Louis, MO).

Virus-specific Antibody. CMV-specific antibody was measured in a solid phase radioimmunoassay (RIA; 13), using appropriate concentrations of CMV-infected or mock-infected cell lysates (CMV(AD169) CF antigen or control antigen; Flow Laboratories, McLean, VA), test sera or culture supernatants, rabbit anti-human immunoglobulins, and ^{125}I-labeled Protein A. Antigen was coupled to polyvinylchloride microtiter plates and individual wells were cut out and counted in a gamma counter. A negative sample was defined as one that bound no more radioactivity to CMV-infected lysates than to mock-infected lysates i.e., had a binding ratio of 1.5 or less. The sensitivity of this RIA was ~200-fold greater than that of a standard immunofluorescence assay for CMV antibodies (14). EBV-specific antibody was measured by immunofluorescence (3); all but one donor (GG) was seropositive for EBV.

Results

Induction of Plaque-forming Cells by CMV. Peripheral leukocytes from CMV seronegative donors were depleted of T cells and cultured with EBV, mock-infected control HFF preparations, live Towne strain CMV at a m.o.i. of 0.02, or irradiated Towne strain CMV at the same concentration. After a minimum of 11 d in culture, the numbers of Ig-secreting cells were measured in a reverse hemolytic plaque assay (Table I). Both live and irradiated CMV and live EBV induced more plaque-forming cells than did the controls, although at this multiplicity of infection the CMV was not as potent a stimulator as EBV.

Immunoglobulin Produced in Response to CMV. More precise analysis of the response to CMV was made by miniaturizing cultures, increasing the m.o.i. to 0.5 and measuring Ig released into supernatants between the 7th and 14th days

TABLE I

Induction of Plaque-forming Cells by EBV and CMV

Donor	Initial composition of culture			Days in culture	Plaques per 10^6 cells		
	E-Rosettes	Esterase +ve	"B Cells"		EBV	CMV	HFF control
		%					
AH	14	35	51	11	104,880	6,500*	7,940
				13	73,250	27,800*	6,500
JD	19	30	51	12	100,000	1,900‡	2,940
				14	104,120	24,460‡	360
GM	18	15	67	12	32,350	4,332‡	1,370
				15	23,040	3,479‡	1,440

* Live CMV.
‡ Irradiated CMV.

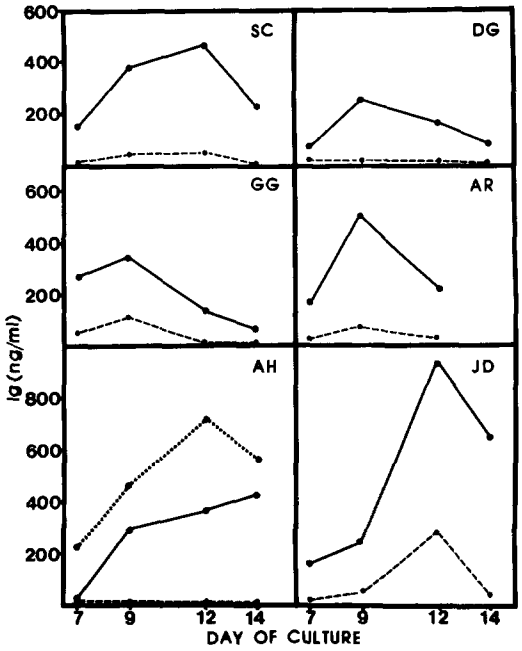

FIGURE 1. Total Ig in cultures from six donors. Cells stimulated with: (●——●) irradiated Towne strain CMV; (●– – –●) irradiated HFF control; (●•••••••●) EBV.

(Fig. 1). Cells from all donors produced immunoglobulin in response to CMV, although the time course of the response was variable. There was no significant difference in the amount produced in response to live or irradiated Towne strain CMV, or to the same equivalent multiplicity of irradiated low passage CMV (Fig. 2). However, cells from some donors died after exposure to infectious low passage virus (not shown). Approximately ⅓ of the total Ig produced was IgM. No CMV-specific antibody could be detected by RIA in the supernatant media, whereas supernatants of cultures derived from three seropositive donors had a binding ratio of >2.

T Cell Dependence of the Response to CMV. A comparison was made among the response of unseparated and T cell–depleted cultures to EBV, a T-independent B cell activator (5, 15), PWM, a T-dependent B cell activator (15), and live CMV (Table II). The responses to PWM and to mock-infected HFF preparations were

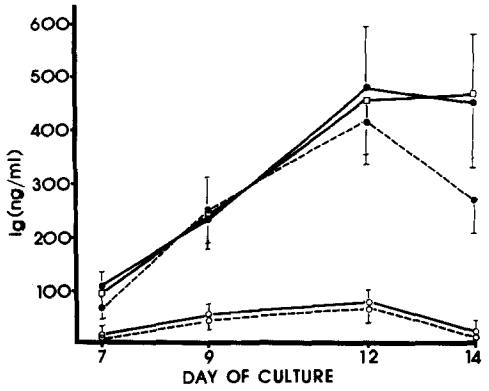

FIGURE 2. Comparison of Ig response to live and irradiated CMV. (●– –●) Live Towne strain CMV; (●——●) irradiated Towne strain CMV; (□——□) irradiated low passage CMV; (○– –○) HFF control; (○——○) irradiated HFF control.

TABLE II

Ig (ng/ml) Produced by Unseparated and T-depleted Cultures

Stimulant	Cell composition	Days in culture			
		7	9	12	14
PWM	Unseparated	435	768	974	307
	T-Depleted*	47	19	11	4
EBV	Unseparated	122	211	154	32
	T-Depleted	176	428	513	215
DMV	Unseparated	107	180	433	158
	T-Depleted	237	397	618	380
HFF Control	Unseparated	142	69	171	57
	T-Depleted	71	11	29	21

* Cultures contained <0.5% T cells.

markedly reduced in cultures depleted of T cells. In contrast, the responses to EBV and CMV were increased.

Discussion

The work in this paper indicates that CMV can induce human lymphocytes from CMV seronegative donors to produce immunoglobulin. None of the Ig produced was detectably CMV-specific; this was in contrast to the small amount of CMV-specific antibody that was produced by leukocytes cultured from CMV seropositive donors. At least ⅓ of it was IgM. The amounts produced were of the same order of magnitude as those induced by EBV or PWM. We conclude therefore that the response to CMV is not an amplified anamnastic response, but rather a true nonspecific polyclonal activation of B cells.

The immunoglobulin response to CMV, unlike that to EBV (5) is not abrogated by irradiation of virus. Neither is it limited to laboratory strains of virus. The contrast in the effects of T cell depletion clearly distinguishes the response to PWM and HFF controls from that to EBV and CMV. PWM is a T-dependent mitogen and the reduction in the response of T-depleted cultures to the HFF control, suggests that this "background" level of Ig synthesis results from a response to T-dependent cellular or FCS antigens. The failure of T cell depletion

to reduce the response to CMV suggests that CMV, like EBV, is a T-independent polyclonal B cell activator. Although the amounts of Ig produced by T-depleted cultures increased with the numbers of B cells present, the increase was not directly proportional for either CMV or EBV. This may reflect a deterioration in culture conditions (17); certainly, the exchange and effective dilution of culture medium four times during the assay period may have had deleterious effects. However, it is also possible that changes in B cell/monocyte ratios, that are attendant on T cell depletion, may have profoundly influenced virus/leukocyte interactions (16, 18).

Hypergammaglobulinemia, cryoglobulinemia, and autoantibody production are features of CMV-IM and post-perfusion syndrome (6, 7). The ability of CMV to stimulate polyclonal Ig synthesis provides a provocative explanation for these phenomena and identifies what may be a pathogenic event common to CMV and EBV mononucleosis. It merits further study as a possible indirect cause of the changes in T cell function in mononucleosis. In addition, in view of the association of CMV with AIDS (19), it might be investigated as a contributor to the abnormalities of B cell function that may occur in this syndrome (8).

Summary

Human cytomegalovirus is shown to be a nonspecific polyclonal B cell activator. The B cell response is independent of virus replication and requires little, if any, T cell help.

We thank Ann McNicol for excellent technical help and Herb Houck for providing fibroblast cultures.

Received for publication 15 September 1983. and in revised form

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