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EXPRESSION OF HUMAN CYTOMEGALOVIRUS IMMEDIATE EARLY PROTEIN IE1 IN INSECT CELLS: SPLICING OF RNA AND RECOGNITION BY CD4+ T-CELL CLONES+

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SUMMARY. Recombinant baculoviruses containing the unspliced gene (Bac-IE1) and a truncated cDNA (Bac-EX4) of the immediate early protein 1 (IE1) of human cytomegalovirus (HCMV) were constructed. The recombinant proteins IE1 and EX4 were expressed in Sf 9 insect cells. Immunoblot analyses using a specific monoclonal antibody or human sera from HCMV seropositive subjects revealed that the IE1 protein had an apparent molecular mass of 71 kDa which was similar to that observed in both HCMV infected human fibroblasts and infected or transfected human astrocytoma cells. Furthermore, HCMV-specific CD4+ T cell clones proliferated in the presence of IE1 or of EX4 used as a control, and appropriate antigen presenting cells. Our data on the IE1 gene provide evidence that two introns can be properly spliced out in baculovirus infected insect cells. The expressed proteins should be useful in further studies on the immune response to the virus.

Human cytomegalovirus (HCMV) is a beta-herpesvirus that establishes a persistent infection in immunocompetent individuals and is a causative agent of morbidity and mortality in immunocompromised hosts, particularly those undergoing organ transplantation or with AIDS (1, 2). Expression of HCMV major immediate early (IE) proteins is the first, obligatory step in the virus life cycle. Genes for these proteins, located between map unit 0.739-0.751, are all under the control of a single powerful enhancer/promoter (for reviews see 3 and 4). The most abundant IE protein (IE1) is a phosphoprotein of Mr 72,000 which is translated from a mRNA containing 4 exons (EX1, EX2, EX3, EX4), the first of which is non-coding.

In addition to their importance in virus replication, IE proteins carry immunogenic determinants of both humoral and cellular immunity (5, 6). The importance of cellular immu-

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nity to HCMV has been demonstrated by several studies which show that the development of specific cytotoxic T lymphocytes (CTL) correlates with recovery from disease (7, 8). Further evidence for a central role of cell-mediated immunity was recently obtained by restoration of viral immunity in immunosuppressed patients following transfer of $CD8^+$ T cell clones (9, 10). Cell-mediated immunity to IE1 is also important as we have shown that the T cells precursor frequency of IE1 specific $CD4^+$ T lymphocytes in healthy seropositive donors was high (11). $CD4^+$ responses certainly must play an important role as shown in many other systems (reviewed in 12) since these cells are involved not only in MHC class II restricted cytotoxicity (13), but also are a source of cytokines which affect B cell antibody secretion, CTL differentiation and macrophage differentiation. This raises the question as to the possible role of $CD4^+$ T cell responses in controlling active infection and/or maintaining the virus-host balance during HCMV persistence in man.

In order to study $CD4^+$ T cell responses to HCMV IE1 in the context of HLA class II antigens, we developed baculovirus vectors which expressed IE1 proteins and tested the reactivity of these recombinant proteins with HCMV-IE specific $CD4^+$ T cell clones developed in our laboratory. Construction and expression of the recombinant baculovirus carrying the unspliced IE1 gene containing 2 introns showed that both introns were correctly spliced out in insect cells. We also described construction and expression of a baculovirus containing only Exon 4 of the IE1 gene as a control.

MATERIALS AND METHODS

Cells and virus: MRC5 and Astrocytoma (ATCC, U373MG, designated here A0) cells were grown in BME and RPMI medium (Gibco, Cergy, France), respectively. The media were supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 1mM Na Pyruvate, 200U/ml penicillin, and 100μg/ml streptamycin (RPMI-FCS). A2 cells, stably transfected with the Towne strain HindIII C fragment, were a gift of R. LaFemina (Merck Sharp and Dohme, West Point, PA, U. S. A.) and were established by co-transfecting plasmid pRL103 (14) and a gene for neomycin resistance (ratio 5:1). Spodoptera frugiperda cells (Sf9, ATCC N° CRL1711) were grown in TC100 medium supplemented with 10% FCS. The Towne strain of HCMV was a gift from M. P Landini (Instituto di Microbiologia, Bologna, Italy). Infections of confluent monolayers of A0 or MRC5 cells were performed at 5 pfu/cell for 24 hrs before cell lysis and Western blots analysis (see below).

Construction of recombinant baculoviruses expressing IE1 and EX4 (Fig 1)

Production of the transfer vectors pGmAc34-IE1 and pGmAc34-EX4: pRL103 plasmid which contains the HindIII C fragment of the HCMV Towne strain was a generous gift from R. LaFemina (15). A fragment of genomic DNA including exons 2,3 and 4 of the IE1 gene was derived by the polymerase chain reaction (PCR) using Taq polymerase (Promega,Coger,France). Primers were synthesized according to the published sequences of IE1 cDNA (16) with additionnal SmaI(↓) sites for cloning into plasmid pGmAc34. The sequence of primers were follows: P1 = 5'.ATTAATCCCGGGATGGAGTCCTCTGCCAAGAG. 3'; P2 = 5' ATATCCCGGGTTACTGGTCAGCCTTGCTTCTA 3'. Two hundred ng of pRL103 were annealed to 50 pmole of each primer and submitted to 30 cycles of amplification as follows: 95°c/ 90 sec; 55°c/ 90 sec; 72°c/ 120 sec. A fragment of the IE1 gene corresponding to exon 4 was generated by the same procedure except that an ATG codon was introduced at the 5'-end of the primer P3 whose sequence were as follows: P3 = 5'. TCTCCCGGGATGGTCAAACAGATTAAGGT 3'. The PCR products were isolated from agarose gel with Qiaex DNA extraction kit (Promega,Coger, France), made free of Taq polymerase by treatment with proteinase K (50 μg/ml in 10 mM Tris buffer pH 8.0, 5 mM EDTA, 0.5% SDS) and blunt ended with SmaI endonuclease. Plasmid pGmAc34 is a modified vector in which the polyhedrin start codon has been mutated (ATG to ATT) and a unique SmaI cloning site introduced at a deletion between nucleotide

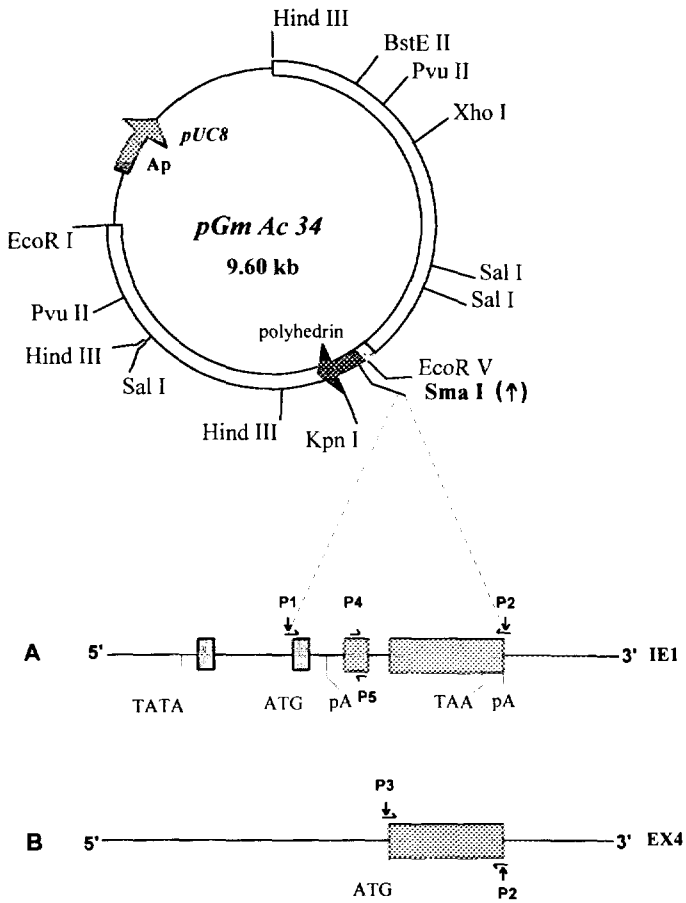

Figure 1. Construction of the pGmAc34-IE1 and pGmAc34-EX4 baculovirus expression vectors. (A) pGmAc34-IE1 contains all the genomic DNA of the IE1 gene including introns and (B) pGmAc34-EX4 contains a DNA fragment corresponding to exon4 of IE1 ORF. P1 to P5 indicate the forward and reverse primers used in the PCR amplification of IE1 and EX4.

+44 and +462. Translation of the cloned genes starts from the natural ATG in the P1 primer for IE1 or from the ATG added at the P3 primer for EX4. Both end with the natural TAA stop codon in the P2 primer. Conditions for ligation and selection of transformants were as described (17). Clones with proper orientation were submitted to Sanger dideoxysequencing (18).

Isolation of Bac-IE1 and Bac-EX4 recombinants: Spodoptera frugiperda cells (Sf 9, ATCC# CRL1711) were transfected with recombinant plasmid pGmAc34-IE1 or pGmAc34-E4 (5μg) and Autographa californica nuclear polyhedrose virus (AcNPV) DNA (1μg). Liposomes (Dotpa, Boehringer, Mannheim, FRG) were used for the cotransfection of Sf 9 cells at 50% confluency. Cells were grown in TC100 medium (Gibco, Cergy, France) supplemented with 10% fetal calf serum (FCS). Five days after transfection, viral progeny was collected, diluted, and recombinant viruses selected for the absence of nuclear viral occlusion bodies (OB^-) in lysis plaques with Sf 21 cells (19). Four rounds of plaque purification generated wild type-free Bac-IE1 and Bac-EX4 recombinant viruses. Bac-IE1 and Bac-EX4 recombinants were amplified to 10^8 PFU/ml and Sf 9 cells infected at MOI:10 for protein production. The same culture conditions were used to obtain wild type AcNPV infected Sf 9 cells (Bac-WT).

Western blot analyses: Cells were pelleted by centrifugation, suspended in distilled water and diluted in disruption buffer (50 mM Tris pH 6.8, 1% SDS, 10% glycerol, 5% β-mercaptoethanol). Aliquots corresponding to $5x10^5$ cells were heated for 4 min at 95°C, loaded onto 10% acrylamide gels and electrophoresed (SDS-PAGE) according to Laemmli

(20). Prestained molecular weight standards (Gibco) were run in the same gel. Following electrophoretic separation, proteins were transferred to nitrocellulose sheets (Hybond C Super, Amersham). Blots were blocked for 5 min in blotto (5% skim milk, 0.2% NP40 in PBS pH 7.4), then incubated overnight at 4°C in fresh blotto containing appropriate dilutions of either human sera from HCMV seropositive donors or mouse monoclonal antibodies (McAb E13, Biosoft; McAb DDG9, Dako). After three 10-min washes in 0.2% NP40 in PBS, the filters were incubated with peroxidase-conjugated rabbit anti mouse IgG or peroxidase-conjugated goat anti human IgG (RAM/PO, GAH/PO, Dako) in blotto for 1 hour. After washing in 0.2% NP40-PBS, the blots were developed by incubation in PBS containing 0.5mg diaminobenzidine (DAB), and 0.05% H2O2.

RT-PCR analysis of IE-RNA

Isolation of RNA: Total cellular RNA was isolated from cells grown in monolayer by direct lysis in the culture flask as described (21). RNA was prepared from 5 X 10^6 cells of each of the following samples: uninfected MRC5 cells, HCMV-infected MRC5, A0 or A2 astrocytoma cells, and Sf 9 cells infected with Bac-IE or Bac-WT.

First strand cDNA synthesis for PCR: A cDNA cycle kit from Invitrogene (British Biotechnology, Abingdon, U.K.) was used to generate first strand cDNA from total RNA (5 μg) with oligo dT primer. Half the cDNA was used as a template in PCR amplification under conditions similar as those described above whatever couple of primers was used. In order to show that splicing had occurred in the message, PCR reactions were carried out with primers P4 and P5 whose relative locations are indicated in figure 1. The sense P4 primer (5' AGTCAGCTGAGTCTGGG 3') corresponds to IE1 cDNA nu136 to nu152. The P5 primer (5' CGAGTTCTGCCAGGACATC 3') represents the antisense strand of the IE1 cDNA sequence nu238 to nu258.

Generation of HCMV specific CD4$^+$ T cell clones: Antigens for T cell stimulation were prepared as follows: confluent A0 or A2 cells (confluent 75 cm^2 flask) were washed twice with PBS, detached by scraping and sonicated in a small volume of PBS. The suspension was then centrifuged at 2000 g and the supernatant was collected and frozen at -70 °C until further use.

T cell clones were prepared in 96-well round-bottom microplates (Falcon, Becton Dickinson, New Jersey) as follows: Peripheral blood mononuclear cells (PBMC) (3-5 X 10^3) from healthy, HCMV seropositive blood donors were incubated in the presence of 6 x 10^4 irradiated (2000 R) autologous PBMC (APC) and an appropriate concentration (predetermined by serial dilution) of A2 lysate in RPMI-HCMV$^-$ (RPMI + aminoacids, antibiotics, +10% serum from a pool of seronegative blood donors (J.Boy, REIMS, France) in a final volume of 100 μl. A week later, cells were fed with 100 μl of RPMI-HCMV$^-$ medium containing 20 U/ml of IL-2 (a gift of Roussel Uclaf, France). Cells from wells positive for growth were transferred into 1 ml of RPMI-HCMV$^-$ + 20 U/ml IL-2, cultured in 24 well plates (Falcon, Becton Dickinson, New Jersey), then tested in a proliferation assay (see below). After several stimulations with Ag in the presence of autologous APC, T cells were subcloned at 10, 3, or 1 cell per well in 96-well round bottom microplates, in the presence of irradiated allogeneic PBMC, 1μg/ml PHA (Difco, OSI, Paris, France) and 20U/ml IL-2. They were maintained in culture in RPMI-FCS + IL2 and restimulated every 3-4 week with irradiated allogenic PBMC + PHA + IL2.

T-cell specificity assay: T cells (20 X 10^3) were incubated in round bottom microtiter plates, in triplicate, in the presence of 10^5 irradiated autologous or HLA-matched PBMC and various dilutions of Ag in a final volume of 100 μl of RPMI-FCS. On day 3, the cultures were pulsed overnight with [^{3}H] TdR (Amersham) (1 μCi/ well). The [^{3}H] TdR incorporation was determined by liquid scintillation in a beta counter.

RESULTS

Expression of IE1 and EX4 in Sf 9 cells

Several clones of pGmAc34 plasmids containing PCR products of IE1 or EX4 were obtained. pIE1 plasmids contained the expected 1769 bp insert and pEX4 the expected 1200 bp insert. Sequencing of pIE1 showed a single nucleotide change which affected neither the open reading frame nor the nature of the corresponding amino acid residue with respect to the sequence of the Towne HCMV strain. Sequencing of pEX4 clone showed no discrepancy with the viral sequence.

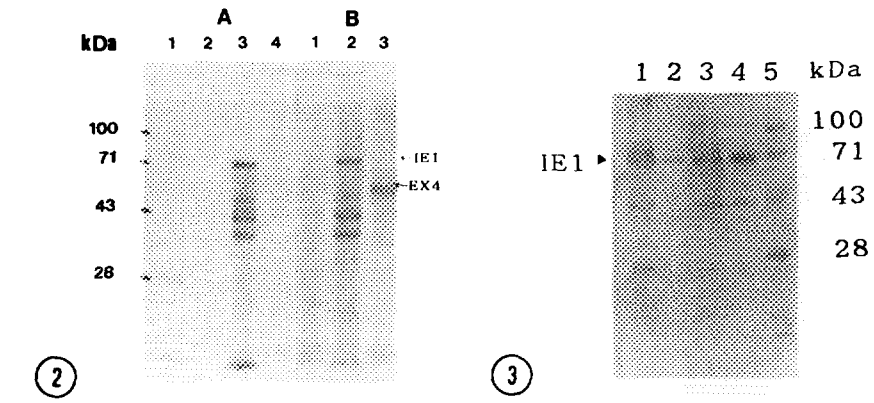

Figure 2. Immunoblot of IE1 and EX4 recombinant proteins with E13 monoclonal antibody (A) or a CMV-positive human serum (B). Sf 9 cells were infected with wild type virus Bac-WT (A,2 and B,1), Bac-IE1 (A,3 and B,2) or Bac-EX4 (A,4 and B,3) recombinant viruses. Lysates were subjected to SDS-PAGE and transferred to a nitrocellulose membrane as described in *Materials and Methods*. Standard molecular weights were as indicated in lane 1.

Figure 3. Immunoblot with McAb E13 of IE1 protein expressed by: (1) HCMV infected astrocytoma cells (A0); (2) IE-transfected astrocytoma (A2); (3) HCMV infected fibroblasts (MRC5) and (4) Bac-IE infected insect cells (Sf9). Standard molecular weights were as indicated in lane 5.

Expression of IE1: To study the biochemical properties of recombinant IE1, cells were infected with Bac-IE1 for 48h, lysed and an aliquot equivalent to $5x10^5$ cells was analysed on immunoblots using the McAb E13. An immunoreactive band with a relative molecular mass of 71000 was observed in cell lysates (Fig 2A, lane 3). The same pattern of reactivity, including bands with higher mobilities, was observed with either McAb E13 or McAb DDG9 (not shown) and with human sera (Fig 2B, lane 2). Mock-infected or Bac-WT-infected cells did not show any band. In order to compare the IE1 protein expressed by A2 cells and by Bac-IE1 infected Sf9 cells with that expressed by cells infected with HCMV, MRC5 cells and A0 cells were infected with HCMV and harvested 24 hours later. The results (Fig 3) show that the IE1 expressed by HCMV either in A0 astrocytoma (lane 1) or in the MRC5 fibroblasts (lane 3) had the same mobility as that expressed by A2 cells (lane 2) and by Sf9 cells infected with Bac-IE1 (lane 4). We further verified whether IE1 mRNA might be different in length in the transfected or the infected systems. In order to detect any minute differences in length between mRNA, IE1 transcripts were generated by RT-PCR 48h after infection and submitted to a high resolution agarose gel electrophoresis. As shown in figure 4, all PCR products, whether generated from stably transfected astrocytoma cells, Sf9 cells infected with recombinant baculoviruses or from human fibroblasts or astrocytoma cells (not shown) infected with HCMV, comigrated in agarose gels at a position (1495 bp) corresponding to the size expected for amplified full length IE1 cDNA. Figure 5 shows that PCR reactions using either the P1-P5 (Fig.5B) or the P4-P2 (Fig.5A) sets of primers, gave rise to 265 bp and 1346 bp fragments, respectively, that migrated at the positions expected for the sizes of spliced transcripts. When genomic DNA (pRL103) was used as a template, longer products were amplified (379 and 1546 bp), suggesting that in transcripts, exon 2 was spliced to exon 3 and exon 3 to exon 4.

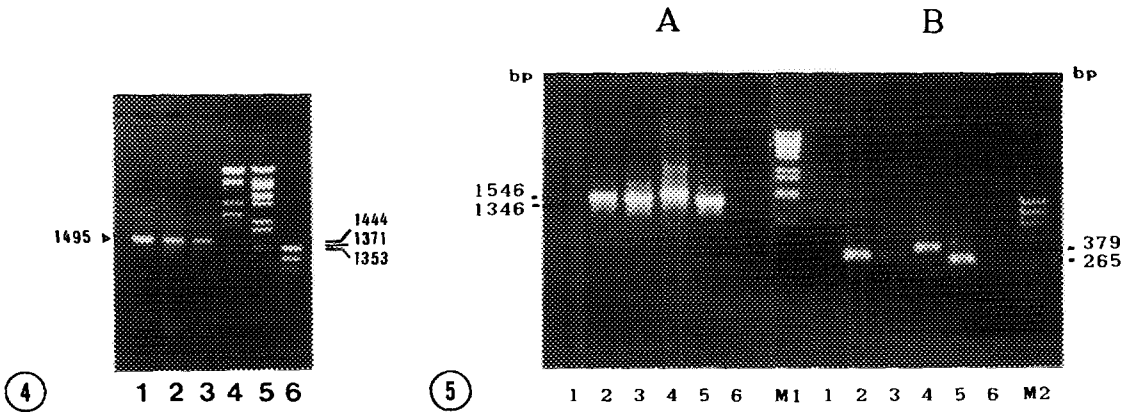

Figure 4. Agarose gel electrophoresis of RT-PCR products using P1 and P2 primers. Total RNA were obtained from : (1) Bac-IE1 infected Sf 9 cells, (2) IE-transfected astrocytoma cells (A2) and (3) HCMV (Towne) infected fibroblasts (MRC5). DNA markers were as follows:(4)λ Acc1, (5) λ BstE II, (6) ϕX174 Hae III.

Figure 5. Agarose gel electrophoresis of RT-PCR products using either P4-P2 (A) or P1-P5 (B) couples of primers. Total RNA (1, 2, 3, 5, 6) and DNA (4) were obtained from: (1) A0 astrocytoma cells, (2) IE-transfected astrocytoma cells (A2), (3) Bac-IE1 infected Sf9 cells, (4) pRL103 plasmid, (5) HCMV-infected A0 astrocytoma cells, (6) Bac-WT infected Sf9 cells. DNA standards were: λBstEII (M1) and ϕX174 HaeIII (M2).

These data indicated that mRNA splicing events were properly carried out in baculovirus infected Sf 9 cells.

Expression of EX4: To examine EX4 expression, Sf 9 cells were infected with Bac-EX4 viruses and tested for reactivity with both high titer anti HCMV human sera and with McAb E13 after protein extraction. None of the antibodies reacted with proteins from uninfected cells or Bac-WT-infected cells. The recombinant EX4 product, with an apparent molecular mass of 48 kDa, was recognized by human serum (Fig 2B, lane 3), whereas McAb E13 did not react (Fig 2A, lane 4). This is in accordance with the specificity of E13 for an epitope localized at the NH2-terminal end of IE1 encoded by exon 2 (22).

T cell proliferative response to IE1 and EX4

Several T cell clones were obtained from three different HCMV seropositive donors. All were $CD4^+$ (data not shown). They were tested in lymphoproliferative assays using IE proteins expressed by Bac-IE1 or Bac-EX4. Figure 6 shows the results obtained with one representative clone from each donor. All clones proliferated in the presence of lysates from A2 cells, but not from A0 cells. This suggested that they might be specific for an IE protein(s) encoded by the Towne Hind-III C fragment of the HCMV genome. A strong proliferative response was seen also in the presence of the Bac-IE1 and Bac-EX4 recombinant proteins but not in the presence of Bac-WT protein extracts. A stronger response was consistently observed with Bac-EX4 compared to Bac-IE1. Inhibition assays using specific monoclonal antibodies for HLA DR, DP, or DQ showed that all 3 clones were HLA-DR restricted (data not shown). These results indicated that the proteins IE1 and EX4 produced by baculovirus infected cells were functional in T cell responses.

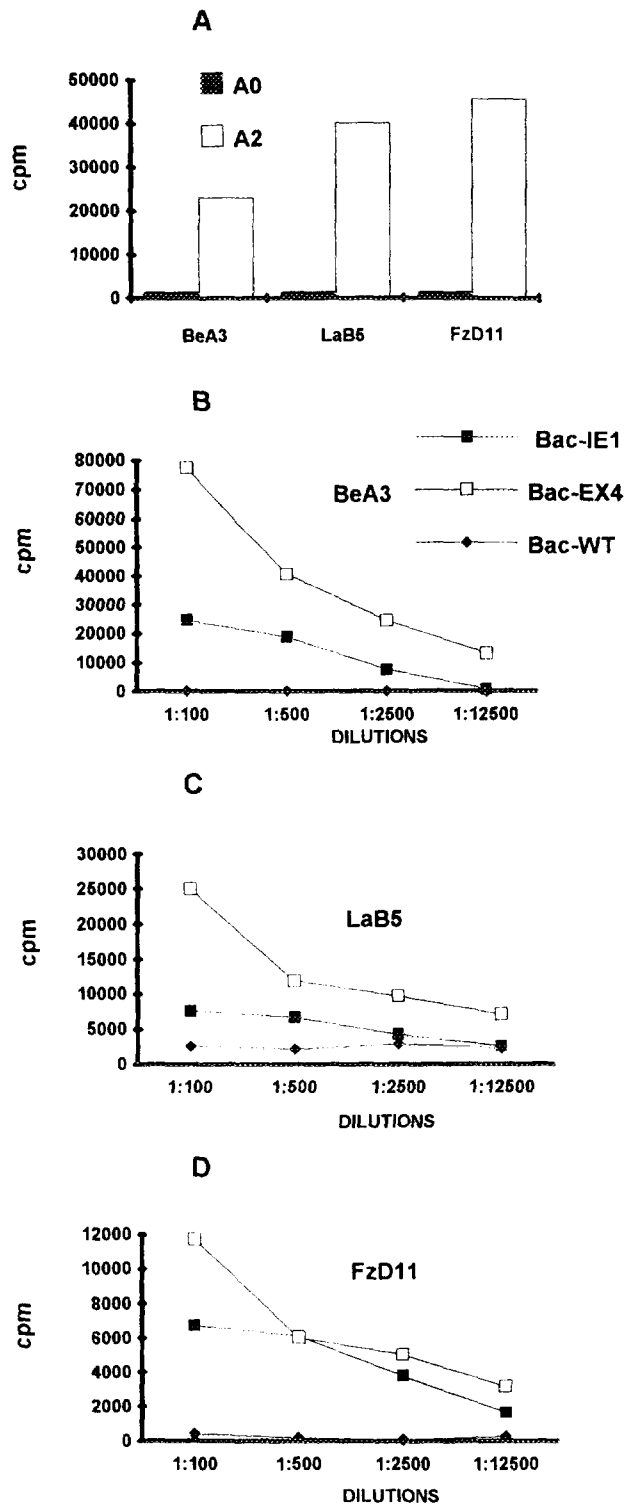

Figure 6. IE1 specific proliferation of T-cell clones. Cells from different clones (BeA3, LaB5, FzD11) were cultured in the presence of irradiated PBL and standardized dilutions of lysates from A2 cells and A0 cells (A) or baculovirus-infected Sf9 cells (B, C, D).

DISCUSSION

The objective of the present study was to develop tools with which to study $CD4^+$ T cell recognition of the HCMV-IE1 protein in the context of MHC class II antigens. For this purpose, we constructed baculovirus vectors expressing the IE1 protein in insect cells and analysed the expression and immunogenicity of the recombinant product. The data presented here show that antigens prepared from a baculovirus expression system using intron-containing DNA were recognized by HCMV-IE-specific McAbs and by human sera and induced a specific $CD4^+$ T-cell proliferative response.

Baculoviruses have emerged as an increasingly useful expression system that allows the production of large amounts of recombinant proteins and efficiently performs many of the higher eucaryotic post-translational modifications in insect cells (23). Our present report shows that this system can perform splicing of the intron-containing gene coding for the IE1 protein. This was demonstrated by the analysis of IE1 transcripts using PCR assays across the introns and by the fact that epitopes within recombinant IE1 were recognized by anti EX4 specific T cell clones. Since IE1 and EX4 antigens induced the proliferation of the same DR-restricted T cell clones, it seems likely that splicing induced the expression of proteins with identical amino acid sequences in the T cell recognized epitopes. Splicing of introns was recently reported in baculovirus genes (24). Our data provide evidence that two introns can be spliced out by baculovirus vectors, thereby extending previous findings that insect cells can efficiently process foreign mRNA containing one intron (25, 26). We have shown that the recombinant IE1 protein in either stably transfected human astrocytoma cells or insect cells appeared to correlate in size with the homologous IE1 polypeptide produced by HCMV-infected cells.

HCMV-specific $CD4^+$ T cell clones were generated using A2 astrocytoma cell lysates. Although these cells carry the whole Towne strain HindIII-C fragment which encodes early, as well as IE, proteins, all T cell clones generated were specific for IE proteins, since they all recognized the product encoded by the EX4 recombinant cDNA. The greater proliferation of the T cell clones observed to EX4, as compared to IE1, might be due either to a greater production of EX4, or to more efficient processing of the protein by antigen presenting cells. Alp et al (27) have shown that polyclonal T cells proliferated in the presence of a recombinant Bac-IE1 protein. The present study further shows that IE1 produced by baculovirus is specifically recognized by HLA-DR restricted $CD4^+$ T cells clones derived from normal $HCMV^+$ blood donors. These reagents should be useful in determining IE1 epitopes recognized by clonal $CD4^+$ T cells.

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