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Interferon Gamma Production by Herpes Simplex Virus Antigen-specific T Cell Clones from Patients with Recurrent Herpes Labialis

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SUMMARY

Nineteen herpes simplex virus (HSV) antigen-specific human T lymphocyte clones were established from three volunteers with recent recurrent herpes labialis. All produced interferon gamma (IFN-γ) at titres of 200 to 700 units/ml when cultured *in vitro* with HSV antigen and irradiated peripheral blood mononuclear cells (PBMC) as filler cells. All 10 of those clones whose phenotype was determined were Leu 4^+, Leu 2^-, Leu 3^+. Interleukin 2 alone failed to induce IFN-γ in titres greater than 10 units/ml from these clones cultured at 10^4/0·2 ml/well. However, the effect of different accessory or filler cells on IFN-γ production by clones was quite marked. For example, high titres were produced when irradiated PBMC or plastic-adherent cells (predominantly monocytes) were added and low titres when macrophages and irradiated Epstein–Barr virus-transformed B (EBV-B) cells were added. When tested for HSV antigen-stimulated IFN production alone, the irradiated PBMC and adherent cells produced low titres, but no detectable interferon was produced by the others. However, with higher concentrations of EBV-B cells, low concentrations of IFN-α were occasionally produced. Irradiation strikingly reduced IFN-α production by PBMC. The IFN-α and -γ produced by accessory cells may contribute to total IFN production by priming the production by cloned cells, and acting in synergy with IFN-γ produced by the cloned cells. Alternatively, the effect may be due to the presence of permissive concentrations of other lymphokines such as the interleukins. Interferon production by cloned T lymphocytes in the presence of non-producing macrophages was maximal within 24 h, much faster than with a similar polyclonal system, although attaining lower titres. EBV-B cells from only one of three patients supported antigen-specific lymphocyte activation. Almost all cells of the three cell lines expressed DR antigens, while DS/DC antigens were also expressed on nearly all cells of the antigen-presenting line and, at lower densities, on two-thirds of the cells of the other two lines.

INTRODUCTION

We recently showed that peripheral blood mononuclear cells (PBMC) from patients with recent recurrent herpes labialis (RHL) produced interferon gamma spontaneously when cultured *in vitro* (Cunningham & Merigan, 1983). The producing cells were nylon wool-adherent Leu 3-positive lymphocytes; the same subset also was the predominant producer when interferon gamma was induced *in vitro* with herpes simplex virus (HSV) antigen (Cunningham & Merigan, 1984). We have also shown that monocyte/macrophage D region antigens are necessary for HSV antigen presentation and subsequent interferon gamma production (A. L. Cunningham, T. Y. Basham, M. F. Para & T. C. Merigan, unpublished results).

Multiple intercellular and humoral interactions involving heterogeneous cell types probably

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occur during the 5 to 9 days required to achieve maximum interferon gamma titres after HSV antigen stimulation *in vitro* (Green *et al.*, 1981). Murine and, more recently, human antigen-specific T cell clones have been used to define humoral and intercellular interactions more precisely (Kimoto & Fathman, 1980; Kurnick *et al.*, 1981). In this study we used HSV antigen-specific T cell clones from patients with RHL to confirm some of our findings and examine others aspects of the mechanisms of interferon gamma production. Single cell assays have shown that a surprisingly small proportion (less than 1%) of mitogen-activated mononuclear cells produce interferon gamma (Wiranowska-Stewart & Stewart, 1981), and recently a figure of 0·1% was reported (Martinez-Maza *et al.*, 1984). Interferon gamma titres produced in the HSV antigen-activated system are similar to those produced spontaneously and we suspect the latter represent the result of antigen exposure *in vivo*.

Therefore, it was of considerable interest to determine the proportion of HSV antigen-specific clones capable of producing interferon gamma. Different studies using various methods of cell separation and induction of interferon have suggested that lymphocytes with exclusively or predominantly helper, cytotoxic/suppressor and natural killer phenotypes are the producing cells (Chang *et al.*, 1982; Kasahara *et al.*, 1983; O'Malley *et al.*, 1982). Murine clonal studies suggested that all these types were capable of producing interferon gamma (Morris *et al.*, 1982; Handa *et al.*, 1982; McKimm-Breschkin *et al.*, 1982), while our own studies using several different methods, suggested that Leu 3-positive lymphocytes were the producing cells in RHL (Cunningham & Merigan, 1984). Clonal isolates have been used here to confirm these findings, to compare the kinetics of production with those of polyclonal populations and, by using different populations of accessory cells, to examine the roles of priming with interferon, stimulation by interleukin 2 (IL 2) and other interactions in the slow attainment of maximum interferon titres in polyclonal systems.

METHODS

Patients. Three volunteers from Stanford Medical Center staff were bled initially for establishment of Epstein–Barr virus (EBV)-transformed B (EBV-B) cell lines. Two to 3 months later, long-term T cell lines were established by re-bleeding them 2 weeks after onset of a recurrence of herpes labialis, confirmed by isolation of HSV in human foreskin fibroblast cultures. Thereafter, the volunteers were re-bled at 10 to 14 day intervals to obtain filler cells needed for periodic restimulation of the clones or cell lines.

Antigen. Membrane-associated, heat-inactivated HSV antigen and uninfected Hep-2 cell controls were prepared as previously described (Rasmussen & Merigan, 1978).

Cell separations. PBMC were separated by discontinuous Ficoll-Hypaque density gradients. Plastic adherent monocytes were obtained by the method of Kumagai *et al.* (1979) and cultured for 7 days in 96-well flat-bottom microtitre plates at 10^4 cells/0·2 ml RPMI + 20% human serum/well. At this stage all viable cells were macrophages by criteria previously described (Cunningham & Merigan, 1983). E-rosette-negative (non-T cells) were isolated by rosetting with AET-modified sheep erythrocytes and aspirating the cells remaining at the interface after sedimentation in a Ficoll-Hypaque gradient (Cunningham & Merigan, 1984).

Preparation of EBV-B lymphoblastoid cell lines. Supernatant medium was aspirated from the EBV-transformed and productively infected marmoset lymphoblastoid cell line, B95-8 (kindly provided by Shu Man Fu, New Haven, Conn., U.S.A.) during their exponential growth phase and centrifuged at 75000 *g* for 90 min (after an initial low-speed spin). The pellet containing EBV was resuspended at 1% of the original volume, passed through a 0·8 μm filter, and stored in aliquots at −70 °C (Miller & Lipman, 1973; Epstein & Achong, 1979). E-rosette-negative (E^-) cells (10^6) in 1 ml RPMI + 10% foetal calf serum were added to each well of cluster well plates (Costar 24-well plates) and either 1 ml of filtered B95-8 supernatant medium or 0·05 ml of concentrated medium was added. 12-*O*-Tetradecanoylphorbol-13-acetate was added to a final concentration of 0·3 ng/ml to enhance transformation (Yamaoto & zur Hausen, 1979). As colony growth progressed the cultures were transferred to culture flasks. The pH was carefully maintained by replenishment of medium (Issekutz *et al.*, 1982).

Establishment of T cell lines and clones. PBMC from the volunteers were cultured in cluster (24-well) plates at 5×10^6/well with 2 ml RPMI + 20% HSV-seronegative pooled human serum, 3×10^{-5} M-2-mercaptoethanol, 12 mM-HEPES buffer, 2×10^{-3} M-L-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml) and HSV antigen at a final dilution of 1/10. After 14 days viable cells were counted with fluorescein diacetate (5 μg/ml) and 10^5 cells added to each cluster well together with 5×10^6 autologous irradiated (3300 rads) PBMC (filler cells) and HSV antigen at 1/10 dilution in 1 ml of the above medium and 1 ml of supernatant from a gibbon leukaemia cell line (MLA-144) as a source of IL 2 (Rabin *et al.*, 1981). Alternatively, some T cell lines and clones were maintained with IL 2-containing supernatants from concanavalin A/pokeweed mitogen (Con A/PMA)-stimulated irradiated

human PBMC adsorbed with Sephadex G-25 (Bonnard *et al.*, 1980). The optimum concentration of IL 2 and HSV antigen were determined by assaying antigen-specific lymphocyte proliferation. This process of periodic stimulation with antigen in the presence of filler cells and IL 2 was repeated at 10 to 14 day intervals. In one patient, irradiated EBV-B cells (2×10^5 per well) were used as filler cells in the later stages of maintenance of long-term T cell lines. Four weeks after initiation of the lines, the T lymphoblasts were cloned by limiting dilution at 0·5 cells/well in flat-bottom 96-well microtitre plates (Linbro) containing 0·2 ml of the above medium with the same concentration of IL 2 and HSV antigen at 1/10 dilution. Irradiated PBMC (10^6 per well) or EBV-B cells (2×10^4 per well) were used as fillers. When confluent, cells from positive wells were transferred to Costar wells with irradiated filler cells (5×10^6 to 10×10^6 PBMC or 2×10^5 EBV-B cells), HSV antigen and IL 2 at the same concentrations in 2 ml of medium. Recurrent stimulation was continued every 10 to 14 days and some of the clones eventually transferred to culture flasks.

Subcloning. A clone from patient 1 was subcloned by limiting dilution using the same methods as for the initial cloning. This subclone and other clones from patients 1, 2 and 3 were used for the interferon experiments. By Poisson statistics it was probable that all clones from patients 1 and 2 were derived from a single cell but that one of the seven clones from patient 3 could be derived from more than one precursor (Henry *et al.*, 1981).

Assay for IL 2 activity. The concentrations of supernatants from MLA-144 (50%) or Con A/PMA (3%) stimulated irradiated PBMC used in the maintenance of clones were standardized with an IL 2 microassay using incorporation of [^{3}H]thymidine (New England Nuclear; sp. act. 20 μCi/mmol) at 24 h by an IL 2-dependent cell line, HT-2, as endpoint (Watson, 1979; Gillis *et al.*, 1978).

Assay for lymphocyte proliferation. One μCi/well of [^{3}H]thymidine (New England Nuclear; sp. act. 20 μCi/mmol) was added for the last 18 h of the lymphocyte cultures. Cell-bound radioactivity was assayed using a PHD cell harvester (Cambridge Technology Inc., Cambridge, Mass., U.S.A.) and a Packard liquid scintillation counter. The means of c.p.m. from quadruplicate wells were calculated (percentage deviation from mean was less than 10%).

Assay and characterization of interferons. Culture supernatants were assayed for interferon by plaque reduction in human foreskin fibroblasts using vesicular stomatitis virus for challenge as previously described (Merigan *et al.*, 1966). An NIH standard leukocyte interferon (5000 units/ml) was run with each assay. The interferon species present were determined by assaying residual interferon titres after incubation at pH 2 and with antibodies to interferons alpha (NIH standard no. G-026-502-568), beta (no. G-028-501-568) and gamma (kindly provided by Dr J. Vilček) as previously described (Cunningham & Merigan, 1983).

Staining of lymphocytes for cytofluorographic analysis. Phenotypes of the T cell clones were determined by staining with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies to Leu 4, Leu 2 and Leu 3 and analysing in a fluorescence-activated cell sorter (FACS III, Becton-Dickinson, Sunnyvale, Cal., U.S.A.) as previously described (Cunningham & Merigan, 1984). Expression of DR and DC/DS antigens on the surface of EBV-B cells was determined with FITC-conjugated monoclonal antibodies to DR (L243) and Leu 10 (Becton-Dickinson Monoclonal Antibody Center, Mountain View, Ca., U.S.A.) antigens. L243 antibody recognizes mature DR antigen and a DR subset (α, β_2) (Shackelford *et al.*, 1982) whereas anti-Leu 10 apparently recognizes a gene product of the DC/DS locus (DC1) (Brodsky, 1984).

RESULTS

Antigen specificity of T cell lines and clones and establishment of optimum conditions for their proliferation

A kinetic study of antigen-induced stimulation of the T cell line HSV-T revealed that proliferation was maximal at 5 days after initiation of culture (Fig. 1). It was found that an HSV antigen final concentration of 1/10 was the highest dilution still giving optimal stimulation. All subsequent proliferation assays used these parameters. Specific stimulation of the T cells was obtained in the presence of HSV antigen utilizing either fresh PBMC or EBV-transformed B cells from patient no. 1 as irradiated filler cells (Fig. 2). The data also shows that EBV-B cells were effective fillers at a ratio of 1 : 1 with the T cells, in contrast to the consistent efficiency of fresh irradiated PBMC to function as antigen-presenting cells at a ratio of 100 : 1 with the T cells. EBV-B cells from patients 2 and 3 did not function as antigen-presenting cells at any cell concentration (data not shown). Supernatant alone containing IL 2 gave good proliferation in a short-term proliferation assay but was itself insufficient for long-term growth of antigen-specific cells. A sample of the antigen-specific proliferation of T cell clones derived from the long-term lines of three patients is presented in Table 1. Although their actual proliferation seems low (stimulation index 1·6 to 5·7), it was in the normal range of stimulation seen with the parental cell

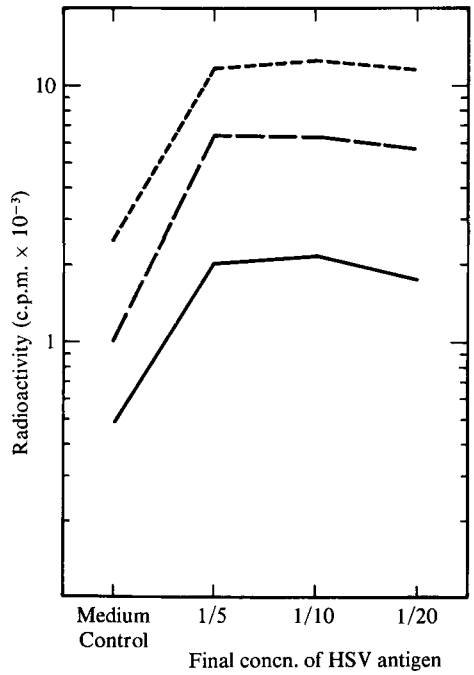

Fig. 1. Proliferative response of HSV antigen-stimulated T cell line (HSV-T) to different dilutions of HSV antigen at 3, 4 and 5 days. T cells at 10^4 per well and irradiated syngeneic PBMC at 10^6/well were incubated for a total of 3 days (——), 4 days (— —) or 5 days (---) with or without HSV antigen as indicated. [^{3}H]Thymidine was added for the final 18 h of incubation.

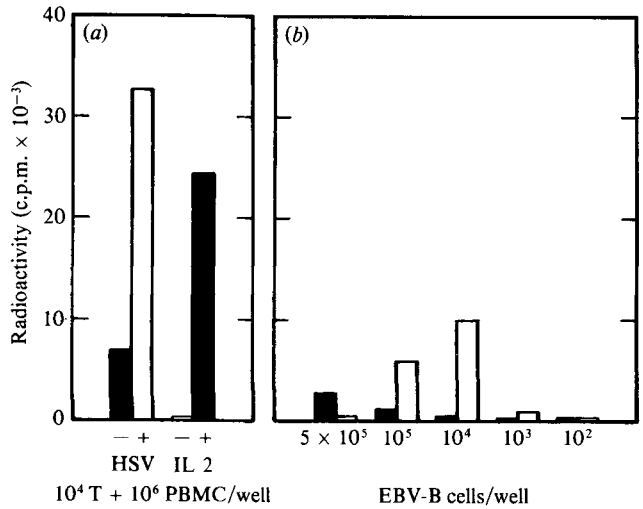

Fig. 2. (*a*) Proliferative response of HSV antigen-stimulated T cell line (HSV-SR) to HSV antigen and interleukin 2 or their controls. T cells at 10^4/well were incubated with irradiated syngeneic PBMC at 10^6/well with or without antigen or medium with or without IL 2 (50% MLA-144 supernatant). (*b*) Effect of varying the EBV-transformed B (filler) cell concentration on the proliferative response of the HSV-SR T cell line to HSV antigen. T cells (10^4/well) were incubated with irradiated syngeneic EBV-B cells at the concentrations shown with (□) or without (■) antigen. [^{3}H]Thymidine was added for the final 18 h of a 5-day incubation.

Table 1. *Antigen-specific proliferation of HSV-T cell clones**

Patient	Clone	Antigen-presenting cell	C.p.m. [^{3}H]thymidine (mean of quadruplicate wells) Medium	+HSV 1/10
1	HSV-SR-A1	EBV-B	861	2554
	HSV-SR-A3	EBV-B	773	1884
2	HSV-T-A3	PBMC	262	996
	HSV-T-A5	PBMC	444	1114
	HSV-T-A6	PBMC	955	1557
	HSV-T-B5	PBMC	2782	5129
	HSV-T-C2	PBMC	241	980
Parental line	HSV-T-cell	PBMC	8249	36366
3	HSV-M-1	PBMC	648	3724
	HSV-M-2	PBMC	817	4622
Parental line	HSV-T-cell	PBMC	5832	29925

* T lymphocytes from clones or long-term T cell lines were incubated (at 10^4 cells/0·2 ml/medium/microtitre well) with irradiated PBMC (100:1 ratio) or EBV-B cells (1:1 ratio) and HSV antigen (1/10 final dilution) for 5 days without IL 2. [^{3}H]Thymidine (1 μCi/well) was added for the last 18 h of culture. Cells were harvested as described in Methods.

lines (stimulation index from 2 to 5), taking into account the lower background when the clones were cultured with irradiated PBMC alone.

Interferon production by T cell clones (Table 2)

Supernatant fluids aspirated 5 days after HSV antigen stimulation from three clones from patient 1, nine clones from patient 2 and seven clones from patient 3 were tested for interferon production. When cultured with irradiated PBMC as filler cells all produced more than 100 units/ml, ranging from 210 to 700 units/ml in the nine fully titrated. However, interferon titres produced by the clones from patients 1 and 2 were much lower when cultured with irradiated EBV-B cells or non-irradiated macrophages. No interferon was produced by unstimulated (control) cultures. Where tested, the interferons produced by the clones were all pH 2-labile and completely neutralized by antibody to interferon gamma. A slight decrease in titre with antibody to interferon alpha was often noted when irradiated PBMC were used as filler cells. Although we could not detect interferon induction by MLA 144 supernatants from long-term T cell lines or clones, IL 2 was omitted from these 19 cultures.

Role of filler cells in the production of interferon (Table 3)

HSV antigen stimulates PBMC to produce a mixture of interferons alpha and gamma with peak titres at 5 to 9 days (90 to 350 units/ml). In preliminary experiments we examined the effect of 3300 rads of gamma radiation on the titres of interferon produced by these cells when cultured at 3×10^5/0·2 ml microtitre well. The titre decreased from 650 ± 178 (mean ± S.D.) to 70 ± 18 units/ml. Interferon alpha was reduced to a greater degree than interferon gamma (unpublished data). Table 3 shows the interferon titres produced by irradiated PBMC and plastic-adherent PBMC (at two concentrations) alone and when combined with cloned T cells. Although the data are incomplete, the optimum ratio of filler cell to cloned T cell for interferon production appears to be 10:1 (or greater) for PBMC and 1:1 for adherent PBMC (monocytes). At these concentrations the cells produce low levels of interferon which must contribute to the total production of interferon by clones as shown in Table 2. Concurrent production of interferon by HSV antigen-stimulated irradiated EBV-B cells (alone) was not demonstrated for the clones of patient 1. However, on one occasion, with 30-fold higher concentrations of these irradiated cells in cluster wells, low titres of alpha interferon were induced with HSV antigen (unpublished data). In a series of studies, we have been unable to induce interferon with HSV antigen from macrophages (at 5×10^3 to 2×10^4 per well).

Table 2. *Interferon production by T lymphocyte clones after HSV antigen stimulation: interferon characterization and phenotyping of clones**

Patient	Clone no. (subcloned)	Filler cell	Interferon titre (units/ml) after: No treatment	pH 2	Antibody to IFN: Alpha	Antibody to IFN: Gamma	Phenotype
1	A1	PBMC	210				
	A3 (+)	PBMC	300	<10	245	<10	
	A1	EBV-B	20				Leu 4+
	A2	EBV-B	15				Leu 2−
	A3 (+)	EBV-B	25	<10	30	<10	Leu 3+
2	A1	PBMC	245				Leu 2−, 3+
	A2	PBMC	700				Leu 2−, 3+
	A3	PBMC	400	<10	280	<10	Leu 2−, 3+
	A4	PBMC	100				
	A5	PBMC	100				
	A6	PBMC	680				
	B5	PBMC	100				
	C1	PBMC	100				Leu 2−, 3+
	C3	PBMC	100				Leu 2−, 3+
	A3	Macrophage	20	<10			
3	1	PBMC	590	<10	625		Leu 2−, 3+
	2	PBMC	600	<10			
	3–5	PBMC	100				
	6	PBMC	620	<10	505		Leu 2−, 3+
	7	PBMC	100				

* Cloned T lymphocytes (10^4/0·2 ml medium/microtitre well) were incubated with irradiated filler cells (PBMC at 100:1 ratio; EBV-B cells and macrophages at 1:1 ratio) and HSV antigen (1/10 final dilution) for 5 days without IL 2. Supernatants were aspirated, pooled from quadruplicate wells and assayed and characterized as described in Methods.

Table 3. *Contribution of irradiated filler cells to clonal interferon production**

Filler cell type	Concentration	Interferon titre: Fillers alone	Interferon titre: Fillers + cloned T cells
PBMC†	10^4/well	<10	25
	10^5/well	20	130
Plastic-adherent	10^3/well	<10	90
PBMC†	10^4/well	60	225
EBV-B†	10^4/well	<10	25
Macrophage‡ (non-irradiated)	10^4/well	<10	20

* Filler cells of different types at various concentrations were irradiated (except macrophages) and cultured in microtitre wells alone or with cloned T lymphocytes (10^4/0·2 ml/microtitre well) and stimulated with HSV antigen (1/10 dilution). Supernatants were aspirated at 5 days and assayed for interferon in parallel.

† Cultured with clone A3 from patient 1.

‡ Cultured with clone A3 from patient 2.

Kinetics of interferon production by T cell clones

Lymphocytes of clone A3, patient 2, were cultured with macrophages in a 1:1 ratio (10^4 per well) and stimulated with HSV antigen in the absence of IL 2. Supernatants were assayed for interferon at 12, 24, 72 and 120 h and antigen-specific lymphocyte proliferation measured at 48 and 96 h. Interferon titres were 15 units/ml at 12 h and reached a maximum of 20 units/ml at 24 h and thereafter remained at this level.

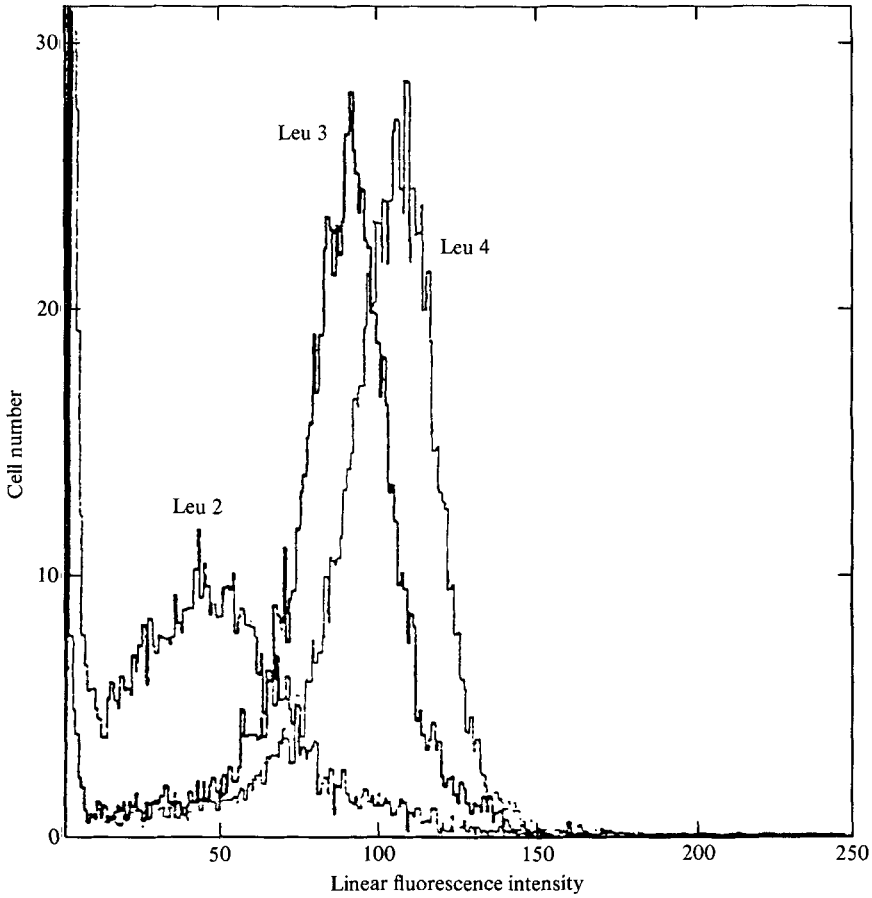

Fig. 3. Determination of phenotype of T-lymphocyte clones by staining with fluoresceinated monoclonal antibodies to Leu 2, Leu 3 and Leu 4 membrane antigens and then analysing (10^4 cells) with the FACS III (Becton-Dickinson).

Phenotypes of interferon-producing T cell clones and D region antigen expression of EBV-B cells

All interferon-producing clones tested were Leu 4^+, Leu 2^-, Leu 3^+ or helper/inducer phenotype (Table 2). The intensity of staining with Leu 3 antibody (shown in Fig. 3) was lower than that usually observed with PBMC (unpublished observations).

The observation that EBV-B cell lines from only one of the three patients supported HSV antigen-stimulated proliferation and interferon production suggested possible differences in antigen presentation and D region antigen expression. The proportion of cells expressing DR and DS/DC antigens and their mean densities as measured by intensity of staining are shown in Table 4. Eighty-five % of all cells expressed DR antigens and 96% of the cells of patient 1 expressed DC, whereas two-thirds of the other two patients' cells expressed DC/DS antigens. In the latter two lines, two distinct populations of positive cells were not observed. The lower proportions of positive cells were due to lower fluorescence intensities (means 105 and 110 fluorescence units) in these two populations compared with the presenting line (mean 170 fluorescence units).

DISCUSSION

This study reports the induction of interferon gamma from HSV antigen-specific human T cell clones. Nineteen clones were established from three patients with a recent recurrence of herpes labialis, at a stage when antigen-specific T lymphocyte activation has been demonstrated

by several parameters (Cunningham & Merigan, 1983; Rasmussen *et al.*, 1974; Shillitoe *et al.*, 1977). All clones were antigen-specific but clonal specificity to the complex antigen used here could include specificities to many HSV proteins and glycoproteins. The interleukin 2 concentrations and culture conditions favoured the development of helper/inducer T cell clones (Kimoto & Fathman, 1980; Kurnick *et al.*, 1981) and indeed all of ten clones characterized were of this subset. Surprisingly, all clones tested produced interferon in moderately high titres (210 to 700 units/ml) after HSV antigen stimulation in the presence of irradiated PBMC as filler cells. In all five cases fully characterized, the interferon produced was gamma. Acid-labile alpha interferon was not detected in significant amounts in this study although we (Cunningham & Merigan, 1984) and others (Balkwill *et al.*, 1983) have demonstrated production of this interferon from human Leu 3-positive T lymphocyte subsets and cell lines. In our hands, IL 2 alone (or with HSV antigen) did not induce a T cell line or two clones from different patients to produce detectable interferon levels (greater than 10 units/ml). Hecht *et al.* (1983) have recently reported studies on interferon gamma production by murine T cell clones which were specific for pigeon cytochrome *c* antigen. There were many similarities with results reported in this present study. Using irradiated spleen cells as antigen-presenting cells, they obtained similar interferon titres and the clones developed were also helper/inducer phenotype (Lyt 1^+, 2^-). However, in contrast to this study, occasional dissociation of clonal proliferation and interferon production was demonstrated by using species variants of the cytochrome *c*. Future studies with purified glycoproteins of HSV 1 and 2 might reveal a similar phenomenon in this system. They also showed the induction of low titres of interferon gamma with IL 2 but their cell concentrations in cluster wells were five times those which we employed. In other studies of long-term T cell lines and clones, IL 2 induction of interferon gamma has not been consistent, although IL 2 often enhanced the effect of other inducers (Handa *et al.*, 1982; Benjamin *et al.*, 1982; Palladino *et al.*, 1983).

The contribution of irradiated filler or antigen-presenting cells to clonal interferon gamma production by antigen-specific T cell clones has not been previously addressed. HSV antigen-stimulated PBMC produce predominantly interferon alpha during the first 2 days in culture and then a mixture of alpha and gamma interferons, reaching a peak at 5 to 9 days. Irradiation has been reported to reduce yields of interferon alpha induced by viral infection of animals (De Maeyer *et al.*, 1969). We showed gamma irradiation (3300 rads) reduced interferon yields tenfold *in vitro*, with a greater reduction in interferon alpha than gamma. However, low titres of interferon were still produced by the filler cells in conditions identical to those of their co-culture with cloned T cells. They must contribute at least an additive amount of interferon. In contrast, EBV-B cells did not produce interferon under parallel culture conditions to their co-culture with clones. At EBV-B cell concentrations optimal for support of clonal proliferation, interferon production by T lymphocyte clones was much less than with PBMC as fillers. This suggests that antigen presentation is not sufficient for production of optimum interferon titres, particularly as these cells express D region antigens and their epitopes (recognized by L243 and Leu 10 monoclonal antibodies) necessary for both proliferation and interferon production. The enhanced production with PBMC or plastic-adherent cells may be due to either production of other monokines (probably deficient in EBV-B cells), priming with interferon (Stewart, 1979) or synergism between gamma and trace amounts of alpha interferons (Schwarz & Fleischmann, 1982). IL 2 production from irradiation-resistant T lymphocytes in the PBMC may occur but is unlikely with the plastic-adherent cells (Inouye *et al.*, 1980). Maturation of the plastic-adherent cells into macrophages was associated with loss of interferon production after HSV antigen stimulation and also lower interferon yields when used as antigen-presenting cell with T cell clones. However, a moderate antigen-specific proliferative response was retained, similar to that with polyclonal nylon wool-purified (NWP) T lymphocytes (at 3×10^5/well) in our previous studies (unpublished data). Future studies of the proportion of interferon gamma-producing cells in association with each type of filler cell will be most interesting.

Macrophages were selected for kinetic studies to ensure that kinetics of clonal production rather than filler cell production of interferon were being determined. Interferon gamma levels reached a peak within 24 h and then remained constant for at least 5 days. However, in a system

using the same macrophages and NWP T lymphocytes, peak levels are attained much later, at 5 to 9 days (Cunningham & Merigan, 1983; A. L. Cunningham, T. Y. Basham, M. F. Para & T. C. Merigan, unpublished results). Hecht *et al.* (1983) reported similar kinetics with antigen-specific murine clones. Mitogen-stimulated murine clones also achieve maximum interferon gamma levels more rapidly than do polyclonal mononuclear cells (Benjamin *et al.*, 1982; Krammer *et al.*, 1983). Presumably the rapid rise to maximum levels reflects the already activated (DR antigen-expressing) state of T cell clones and their synchronous induction by presented antigen, whereas with NWP cells which do not initially express DR antigen, perhaps a process of activation or priming of the various antigen-reactive clones occurs progressively during this 5 to 9 day period. In addition, the cloning process is much more rigorous in excluding non-T cells than a nylon wool column. Retention of some of these cells may provide small amounts of alpha interferon for priming or synergism.

As shown by Issekutz *et al.* (1982), the use of EBV-transformed cells as APCs in human T cell cloning reduced the need for repeated venepuncture. However, we were only able to use one of three patients' transformed cell lines for this function. The reasons for our failure in the two remain to be clarified. All three transformed lines were healthy and had been passaged many times over 3 months making retention of monocytes most unlikely, and EBV-B cells of patient 1 continued to support antigen-specific proliferation over several months without waning. No significant difference in the expression of DR antigens was observed among the lines. Although the two non-presenting lines expressed Leu 10 (DC1) antigens at lower densities (as measured by the lower mean fluorescence intensities) than the presenting line, this seems unlikely to account for a complete inability to support proliferation. A recent study showed some T cell clones recognize antigen in association with SB antigens but the majority recognized DR (Eckels *et al.*, 1983). In our studies several clones and also T cell lines from patients 2 and 3 failed to proliferate, excluding this possibility. Perhaps differences in lymphokine secretion may be partly responsible or transformation may alter the capacity of antigen to associate with D region antigens. Studies to examine some of these alternatives are in progress.

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