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Infection of human thymic epithelial cells by human cytomegalovirus and other viruses: effect on secretion of interleukin 1-like activity

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SUMMARY

Cultures of human thymic epithelial (TE) cells are able to produce a interleukin 1 (IL-1) like activity. This IL-1 activity can be detected either using mouse thymocytes in a traditional IL-1 assay, or using thymic lymphocytes obtained from cases of pediatric cardio-vascular surgery. Production of IL-1 activity by TE cells was found to be maximal between 3 and 4 weeks after culture initiation. Human thymocytes worked best as targets in an IL-1 assay, when these cells were derived from donors younger than 1 year of age. Infection of human TE cells by any of human cytomegalovirus, herpes simplex virus type 2, adenovirus 7, Coxsackie B1, and respiratory syncytial virus led to marked reductions in the ability of these cells to secrete measurable IL-1 activity. In the case of TE cells infected by cytomegalovirus, respiratory syncytial virus, and Coxsackie B1, this abrogation of production of IL-1 activity occurred in the absence of any obvious virus-induced cytopathic effect.

Keywords thymic epithelium cytomegalovirus IL- lymphocytes

INTRODUCTION

It is clear that a variety of viruses can exert profound inhibitory effects on the immune system. However, the mechanism(s) by which many of these viruses induce immune suppression are not clearly understood. Several investigators have demonstrated that a variety of viruses are able to inhibit mitogen-driven lymphocyte blastogenesis *in vitro* independently of infection, and have argued that viruses might be able to impede immune responsiveness on the basis of structural interactions with cells of the immune and/or reticulo-endothelial systems (Fowler *et al.*, 1977; Wainberg, Vydelingum & Margolese, 1983; Copelan *et al.*, 1983).

The thymus is an organ which plays a key role in the development of the immune system during gestation. There is good evidence to indicate that the epithelial cells which are found in the thymus are essential to T cell maturation and differentiation (Stutman, 1978; Haynes *et al.*, 1983). This is a role which may continue into the post-natal period and which may persist until the time at which thymic involution is complete.

Previous research (Oosterom & Kater, 1980; Oosterom, Kater & Oosterom, 1979) has demonstrated that conditioned medium from human thymic epithelial cell cultures contains a soluble factor which can enhance the incorporation of tritiated thymidine ([^{3}H]TdR) into PHA-stimulated mouse or human thymic lymphocytes of young donors. Thus, this activity bears strong biological resemblance to that of interleukin 1 (IL-1), which is usually measured in a mouse thymocyte assay (Gery, Gershon & Waksman, 1971; Dinarello, 1984). Of course, IL-1 is traditionally produced by cells of monocyte–macrophage lineage, and acts on subsets of T helper cells to cause them to produce IL-2, which is directly responsible for T cell proliferation (Smith, Gilbride & Favata, 1980). We reasoned that, were viral infection of the thymus or thymic epithelium to take place, this might affect the ability of this organ to play its essential role in ontogeny. Such infection might conceivably take place either of thymic lymphocytes or of the thymic epithelium itself, and could account for some of the immune suppression which is seen in pediatric populations. We investigated whether viral infection of the thymic epithelium could occur, and the extent to which such infection might interfere with the synthesis and biological activity of the IL-1-like thymic factor described by others.

MATERIALS AND METHODS

Virus

With the exception of the AD-169 laboratory strain of human cytomegalovirus (CMV AD-169), all viruses were isolated from

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clinical specimens of the diagnostic virology laboratory of the Montreal Children's Hospital. Typing of herpes simplex virus (HSV), influenza A virus, adenovirus, and enteroviruses were performed by the Laboratoire de santé publique du Québec, Ste-Anne de Bellevue, Quebec.

Human CMV, strain AD-169, was propagated in mycoplasma-free human embryonic lung fibroblasts (HEL cells), cultured in Eagle's minimum essential medium (MEM) supplemented with 2% fetal bovine serum (FBS). When cytopathic effect (CPE) was far advanced, cells were harvested by scraping with a rubber policeman, resuspended in phosphate-buffered saline (PBS) supplemented with 10% fetal calf serum (FCS) and 50% sucrose phosphate (0·2 M), and frozen in small samples at −70°C. The viral suspension was freed from debris by centrifugation at 1000 *g* for 10 min. Infectious virus titres were estimated by plaque assay (Wentworth & French, 1970). Two recent clinical isolates of CMV were propagated in the same way.

HSV-2, adenovirus type 7, and Coxsackie B1 virus were all propagaged in HEL cell monolayers. Influenza A (serotype H3N2) was propagated on Rhesus monkey kidney cells (MKC) (Connaught Laboratories, Toronto, Canada). Respiratory syncytial virus (RSV) was propagated on a continuous line of human heteroploid epithelial (Hep-2) cells. At the time of maximum CPE, supernatants were collected and cells were harvested by scraping and pooled with supernatants. The suspensions were rapidly frozen and thawed twice, centrifuged at 1000 *g* for 10 min, and the supernatants were frozen in aliquots at −70°C until further use.

Human thymic epithelial (TE) cultures

Pieces of thymic tissue were obtained from pediatric patients, aged 3 months to 6 years, undergoing cardio-vascular surgery at either the Montreal Children's Hospital or at Hôpital Ste-Justine, Montreal, Canada. The capsule of the thymus was excised and the gland was minced into small pieces (approx. 0·3–0·4 mm). The pieces were exhaustively washed, explanted onto 100 mm tissue culture Petri dishes and fed with a growth medium of equal parts of Dulbecco's modified Eagle's medium and Ham's F_{12}, supplemented with Hepes buffer (1 mM), sodium bicarbonate (1·1 mg/ml), porcine insulin (5 μg/ml), prostaglandin E_1 (25 ng/ml), hydrocortisone (5×10^{-8} M), triiodothyrodine (5×10^{-12} M), human transferrin (5 μg/ml), sodium selenite (5×10^{-8} M), and human newborn cord serum (10% v/v) to encourage explant attachment. The explants were washed each day to clear away unattached thymic lymphocytes. Serum was eliminated from the hormonally-defined medium after 2 to 3 days, when explants had attached to the Petri dishes. This discouraged fibroblast growth and encouraged epithelial cell monolayer expansion. In addition, the absence of cells of macrophage or monocyte lineage was confirmed using indirect immunofluorescence and mouse monoclonal anti-leu-M3 antibodies (Becton-Dickinson, Montreal, Canada). Cultures were kept at 37°C in 5% CO_2 throughout. During the first 10 days, explants and monolayers were fed daily; thereafter they were fed at 2–3 day intervals with RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). After 18–21 days of culture, the monolayers were confluent and contained numerous Hassall's corpuscles interspersed throughout. At this point, the cultures of epithelial cells were generally used for purposes of infection. Examination of these 18–21 day cultures repeatedly showed they were free of contaminating monocytes and lymphocytes, as determined by each of morphological criteria, non-specific esterase analysis, and indirect immunofluorescence, using monoclonal antibodies against T lymphocyte antigens. Beyond 5 or so weeks in culture, there was no further expansion of the thymic epithelial cell population, and degeneration of these cultures became apparent, suggestive of thymic involution.

Viral inoculation

Human TE cell monolayers which had been in culture for a minimum of 14 days were inoculated with 0·5 ml of virus suspension for 90 min before removal of inoculum and addition of 5 ml fresh medium. In all cases, a virus plaque-forming unit (pfu): cell ratio of about 1:1 was employed. The medium was changed thereafter and collected every second day. This procedure was also followed for uninfected control cultures.

Immunofluorescence

Cells were harvested by scraping, washed twice in phosphate-buffered saline (PBS), air-dried on glass slides, and fixed in acetone for 10 min at 4°C. Staining for both CMV-early antigen (EA) and CMV-late antigen (LA) was performed by indirect immunofluorescence, using mouse antisera to CMV-EA (1:8) or CMV-LA (1:10) (Biotech Research Laboratories, Rockville, MD), and fluorescein-conjugated anti-mouse IgG (1:50) (M.A. Bioproducts, Walkerville, MD) as described by Wainberg *et al.* (1985). Staining for RSV was also performed by an indirect immunofluorescence assay using bovine anti-RSV (Burroughs-Wellcome, diluted 1:20) and fluorescein-conjugated goat anti-bovine immunoglobulin (Cappel Laboratories, Cochranville, PA). Staining for HSV-2 was carried out by direct immunofluorescence using fluorescein-conjugated anti-HSV (M.A. Bioproducts, diluted 1:20).

Thymocyte stimulation assays

Human thymocytes (thymic lymphocytes) were obtained from the washing of freshly-obtained thymus tissue, separated from tissue debris and blood by Ficoll-Isopaque centrifugation, and used at a concentration of 10^6 ml in mitogen-driven stimulation assays. These were performed in 96-well microwell plates using RPMI-1640 medium supplemented with 10% FCS and PHA-P (Difco Labs, Detroit, MI), diluted 1:500 (Wainberg *et al.*, 1985). In some experiments, interleukin 2 (Boehringer-Mannheim, Dorval, Quebec) was added to the medium at a concentration of 10% (v/v), and conditioned media from cultures of infected and non-infected thymic epithelial cells were employed at various dilutions in a total volume of 0·25 ml RPMI-1640. Tritiated thymidine (New England Nuclear, Boston, MA) was added to the wells for the final 16 h (2 Ci/mmole) or 6 h (35 Ci/mmole) of a total 72 h incubation period at 37°C (1 μCi/well). Four replicate cultures were studied in each experiment, and each assay was repeated at least three times.

In some experiments, a traditional mouse thymocyte bioassay was used to measure IL-1 activity. This was done using PHA, as described above, and a concentration of 10^6 thymocytes per well in RPMI-1640 medium supplemented with 2% FCS (total volume per well 0·25 ml). Purified rat IL-1 was commercially obtained (Genzyme Inc., Boston, MA) and was used as a positive control in some studies.

Table 1. Secretion of interleukin 1 activity by human thymic epithelial cells

Experiment*	Source of Il-1 Activity†	ct/min incorporated‡	*P*§
1	—	621±88	
	Rat IL-1 (1 unit/ml)	22,152±4167	<0·01
	Thymic epithelial cells (10% v/v)	15,467±3105	<0·01
	Thymic epithelial cells (20% v/v)	28,370±5063	<0·05
2	—	1,432±212	
	Rat IL-1 (1 unit/ml)	36,738±5237	<0·01
	Thymic epithelial cells (5% v/v)	2,781±652	NS
	Thymic epithelial cells (10% v/v)	14,257±1631	<0·05
	Thymic epithelial cells (20% v/v)	24,664±2056	<0·01
	Thymic epithelial cells (30% v/v)	27,518±4126	<0·05

* Experiments 1 and 2 involved different cultures of thymic epithelial (TE) cells.

† Supernatant fluids from TE cells were added directly to the culture media of mouse thymocytes.

‡ ± standard deviation.

§ Probability of significant difference from control cultures to which no exogenous IL-1 activity had been added; Student's *t*-test.

RESULTS

Viral infection of thymic epithelial (TE) cells

Some of the viruses tested caused a rapid cytopathic effect in TE cells, and were deemed to be of little interest with regard to long-term effects of viral infection on thymic function.

HSV-2 caused a rapid and extensive ballooning of infected thymic epithelial cells. Culture supernatants yielded infectious virus particles after 2 days as determined by plaque assay on HEL cells. The presence of viral antigens was further demonstrated in infected thymic epithelial cells by indirect immunofluorescence, performed 2 days after infection. Inoculation of adenovirus type 7 also caused a rapid cytopathology in TE cell cultures. Rounding of most cells was apparent by 3 days after infection, and by day 4 the entire monolayer had lifted from the plate. Infectious virus was present in the culture supernatants from day 3, as determined by passage of culture fluids onto HEL cells. In contrast, each of three other viruses utilized did not result in any cytopathic effect whatsoever, even though the fact that infection had occurred could be established by indirect immunofluorescence or other techniques. For example, although CMV infection of TE cultures yielded no CPE over a 21 day period, the cells became positive for both CMV early and late antigens by day 12. At no time were we able to detect infectious virus particles in culture fluids; however, when monolayers were harvested and sonicated, low titres of infectious virus particles could be demonstrated by infection of permissive HEL monolayers.

RSV did not cause any obvious cytopathology, although infectious virus could be recovered from culture supernatants after 7 days, as determined by inoculation of Hep-2 cells. Infected TE cells could be stained by immunofluorescence, using specific anti-RSV antiserum. Similarly, in the case of Coxsackie virus, no CPE could be visualized, yet infectious virus was recovered from culture fluids at 7 days after infection. Finally, influenza virus failed to infect TE cell cultures, as assessed by haemadsorption with a 0·5% suspension of guinea pig red blood cells, lack of infectious virus in culture supernatants, as assayed on MKR cells, and immunofluorescence.

Biological activity of culture fluids from human thymic epithelial cultures

Other investigators have described that cultures of TE cells are able to secrete a IL-1-like activity, which has the ability to support the growth of mouse thymocytes under conditions of exposure to PHA or other lectins (Oosterom & Kater, 1980; Oosterom *et al.*, 1979). The data of Table 1 show that the TE cultures which we studied also have this ability. Furthermore, the capacity of mouse thymocytes to respond to this IL-1-like factor, in the presence of PHA, was dependent on the concentration of this factor which was present.

We have developed an assay which monitors the ability of this TE-derived IL-1 activity to foster the growth of human thymic lymphocytes or thymocytes, obtained from thymic tissue after teasing and washing; generally, this is done within 24 h of surgery. The results of Table 2 show that these thymic lymphocytes are often unable to efficiently respond to mitogenic stimuli such as PHA, unless co-factors are present. Two such co-factors which enable these cells to undergo a proliferative response are purified recombinant rat IL-1 and purified recombinant human IL-2 (Table 2). Mononuclear cells which are derived from paediatric thymus tissue are sometimes able to respond to mitogenic stimulus, even if no co-factors are included in the reaction mixture, particularly when thymus tissue had been obtained from children over the age of 2 years. This may reflect a greater presence of monocytes in the thymic mononuclear population of older donors.

The data of Table 3 indicate that IL-1-like activity was generally not secreted by cultured TE cells until after 2 weeks of culture. Although some activity was present between 2 and 3 weeks after culture initiation, peak levels of production seemed to be present only after 21 days. In most cases the period following 4 weeks of culture saw a diminution in the levels of biological activity present. For this reason, it was decided to infect cell cultures at times as early as but not earlier than 12 days of age, so that the effect of virus on secretion of IL-1-like activity could be studied later on in cases of slow viral infections.

Effect of viral infection of thymic epithelial cultures on production of IL-1 activity

To study the effect of viral infection on the biological activity of conditioned medium from human TE cultures, we co-incubated thymic lymphocytes from pediatric donors with PHA, in the presence or absence of such fluids. Conditioned media were routinely ultracentrifuged to eliminate virus particles, before testing, even though assays with conditioned media that had not been so ultracentrifuged did not yield different results. Elimination of viral infectivity in the case of HSV, CMV, and adenovirus was ascertained by inoculation of supernatant fluids onto susceptible monolayers of cells. The results of Fig. 1 show that the ability of these cultures to continue to produce IL-1 activity was rapidly lost, following inoculation with HSV-2 and to a lesser extent after infection by adenovirus 7. In contrast,

Table 2. Assay of interleukin 1 activity on human thymic lymphocytes

Experiment	Age of thymic lymphocyte donor	Source of exogenous stimulatory activity*	ct/min incorporated†	P‡
1	6 months	—	1,953±242	
		Rat IL-1 (1 unit/ml)	14,628±3162	<0·05
		Human IL-2 (10% v/v)	68,431±7425	<0·01
		Thymic epithelial cells (10% v/v)	23,915±4107	<0·05
		Thymic epithelial cells (20% v/v)	36,107±2758	<0·01
2	5 months	—	2,637±399	
		Human IL-2 (10% v/v)	54,019±6214	<0·01
		Thymic epithelial cells (5% v/v)	14,684±2171	<0·05
		Thymic epithelial cells (10% v/v)	27,750±4578	<0·01
		Thymic epithelial cells (20% v/v)	33,792±5609	<0·01
3	34 months	—	18,937±3152	
		Human IL-2 (10% v/v)	53,758±4376	<0·01
		Thymic epithelial cells (10% v/v)	35,611±5189	<0·05
4	28 months	—	10,734±2534	
		Human IL-2 (1% v/v)	78,925±10587	<0·01
		Thymic epithelial cells (10% v/v)	39,416±6380	<0·05
		Thymic epithelial cells (20% v/v)	34,055±7169	<0·05
5	14 months	—	1,979±506	
		Human IL-2 (10% v/v)	43,008±6194	<0·05
		Thymic epithelial cells (10% v/v)	8,531±1586	<0·05

* A concentration of 10% IL-2 (v/v) corresponds to about 20 half-maximal units of IL-2 activity per ml. IL-1 activity from thymic epithelial cultures was obtained at times of maximal production.

† ± standard deviation.

‡ Probability of significant difference from control culture to which no exogenous stimulatory activity had been added: Student's t test.

Table 3. Production of IL-1-like activity by cultured human thymic epithelial cells

Experiment	Age of thymic epithelial culture*	ct/min incorporated†	P‡
1	—	1,567±259	
	7 days	1,052±357	NS
	14 days	2,374±459	NS
	18 days	6,995±1320	<0·05
	24 days	27,581±3642	<0·01
	28 days	31,667±5218	<0·01
	35 days	11,086±2354	<0·05
2	—	3,178±289	
	10 days	5,430±1243	NS
	16 days	11,456±2731	<0·05
	21 days	35,178±6383	<0·05
	27 days	26,392±4135	<0·05
	34 days	4,105±568	NS
	40 days	2,197±374	NS

* Culture fluids from thymic epithelial cells were kept frozen at −70°C until use.

† ± standard deviation.

‡ Probability of significant difference from control to which no thymic epithelial cell culture fluid had been added; Student's t test.

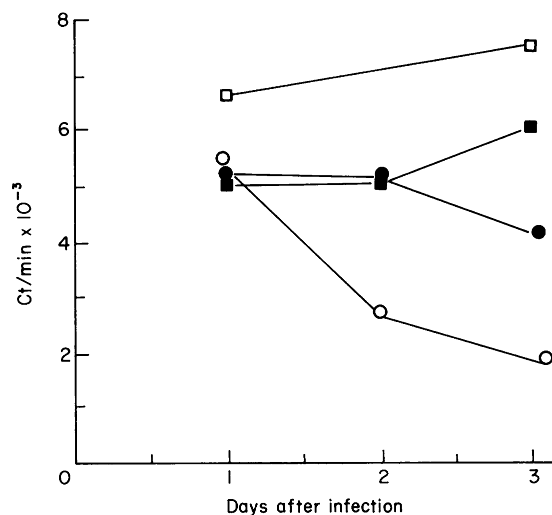

Fig. 1. Effect of conditioned medium from control cultures (□) and from cultures infected by any of influenza A virus (■), adenovirus 7 (●) and HSV-2 (○) on [^{3}H]TdR incorporation into PHA-stimulated human thymocytes. Thymic epithelial cells were infected after 21 days of tissue culture.

attempted infection by influenza A virus had no such effect. The results obtained with HSV-2 and adenovirus 7 indicated that the presence of virus-induced cytopathic effects in the TE monolayers paralleled the appearance of infectious virus particles in the culture supernatants. It is possible that the loss of IL-1-like

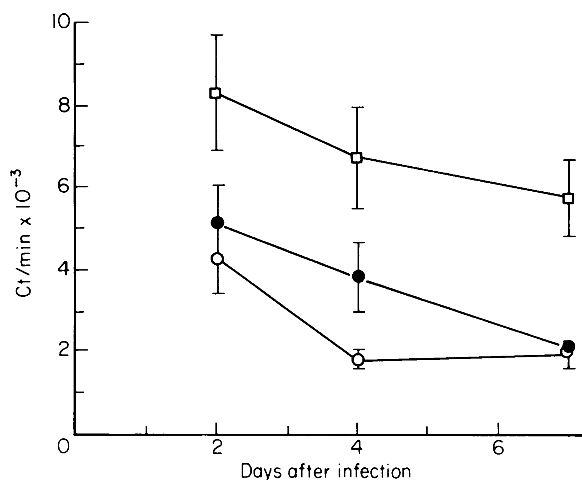

Fig. 2. Effect of conditioned medium from control cultures (□), and from cultures infected by RSV (●) and Coxsackie B1 virus (○), on [^{3}H]TdR incorporation into PHA-stimulated human thymocytes. Thymic epithelial cells were infected after 21 days of tissue culture.

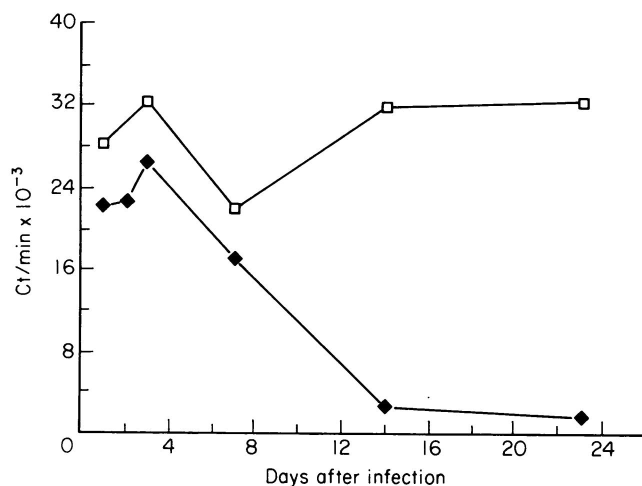

Fig. 3. Effect of conditioned medium from control cultures (□) and from cultures infected by the Ad-169 strain of CMV (♦) on [^{3}H]TdR incorporation into PHA-stimulated human thymocytes. Thymic epithelial cells were infected after 12 days of tissue culture.

activity in these instances could be due to the reduced number of viable cells present after viral infection. Furthermore, potential inhibitory effects could be caused by the release of toxic factors at the time of cell lysis or by the presence of unassembled viral components.

Figure 2 shows the effect of conditioned media from RSV and Coxsackie B1-infected TE cultures in the human thymocyte assay. Infection by either virus resulted in slightly decreased levels of IL-1-like activity by 2 days after infection. Further diminution in activity continued over time, such that no residual activity remained after 1 week. In these cases, the loss of IL-1-like activity was not accompanied by cytopathic effect and was not due to reduced numbers of viable epithelial cells. Infectious progeny virus could be recovered from culture supernatants 7 days after infection.

Following infection by either the AD-169 strain or by either of two clinical isolates of CMV, no cytopathology was detectable in the monolayers at any time over a 3 week period of culture maintenance, in spite of the fact that viral early and late antigens could be detected after 12 days by indirect immunofluorescence. Figure 3 demonstrates the effect of adding conditioned medium from CMV- (AD-169 strain)-infected cultures to our PHA-directed thymocyte stimulation assay. It can be seen that IL-1-like activity could not be detected after 14 days, and that inhibitory factors instead might have been present after that time. Unlike the situation which prevailed after infection by HSV and adenovirus, this was not due, in any way, to a diminution in cell viability or to virus-mediated cytopathic effects. Similar data were obtained on each of two other occasions with TE cells that had been infected by a clinical isolate of CMV.

DISCUSSION

The thymus is a complex, specialized tissue, consisting of epithelial and lymphoid components; this organ plays a crucial role in the maturation and differentiation of T cells (Shortman, 1984; Cantor & Weissman, 1976; Singer *et al.*, 1985; Scollay *et al.*, 1980). In recent years, a number of laboratories, including our own, have succeeded in preparing relatively homogeneous cultures of thymic epithelial (TE) cells for *in vitro* analysis.

CMV is an important member of the herpes virus family that has been associated with immunosuppression (Rinaldo *et al.*, 1980; Pass *et al.*, 1981; Reynolds *et al.*, 1979). In infants suffering from congenital CMV infection, it has been reported that *in vitro* lymphocyte responses to CMV antigens were absent or diminished (Starr *et al.*, 1979). In addition, lymphocyte responsiveness against a variety of specific antigens can be depressed following incidence of CMV mononucleosis (Rinaldo *et al.*, 1980). Clinical isolates of CMV can suppress both natural killer (NK) cell activity and T cell mitogenic responses, as studied *in vitro* (Schrier, Rice & Oldstone, 1986; Rice, Schrier & Oldstone, 1984). CMV has been reported to be able to infect human monocytes as well as T lymphocytes (Rinaldo *et al.*, 1978; Rodgers *et al.*, 1985). Such infection can suppress phagocytic activities and respiratory burst as well as interfere with the ability of monocytes to secrete interleukin 1 (IL-1). In addition, CMV is an important cause of morbidity and mortality in bone marrow transplant recipients and in others whose immune systems do not function adequately, e.g. AIDS patients (Epstein *et al.*, 1985). Thus, CMV has been implicated as a virus which may predispose to immune suppression as well as an opportunistic agent which may cause serious disease in immunocompromized individuals. We have examined the possibility that human thymic epithelium might be susceptible to infection, not only by CMV but by other infectious agents as well, and that such infection might play a role in immunosuppression.

In some cases, the effects of viral infection on TE cell cultures

were devastating. Infection by each of HSV-2 and adenovirus led to rapid cytopathic effects and destruction of the cell monolayer. The fact that these cultured TE cells produced diminished levels of IL-1 activity was considered to be neither interesting nor relevant. Although these viruses can cause congenital infection, neither has been associated with immune suppression in the way that CMV has. In addition, there is no evidence that viral infection, either *in vitro* or in the post-natal period, can lead to widespread destruction of thymic tissue.

Of greater importance, therefore, is the work reported here with CMV, which has been associated with clinical immunosuppression. The fact that neither CMV, nor several of the other viruses studied, led to CPE in the TE cultures studied, while dramatically inhibiting production of IL-1 activity, may be relevant to the mechanisms whereby some viruses achieve suppression of immune response.

The results which we have described for the effects of CMV on TE cells are similar to those which have been reported for the effects of this virus on monocytes and macrophages (Rodgers *et al.*, 1985). Our TE cell cultures were reproducibly shown to be free of contamination by monocytes or macrophages. IL-1, which is produced by TE cells, may function as an essential mediator for mobilization of lymphocytes and for their eventual binding to thymic epithelium (Singer *et al.*, 1986). Other workers have shown that endothelial cells can produce IL-1 activity. It is interesting that CMV can also replicate in such tissue (Ho *et al.*, 1984).

Although the viral infection of thymic lymphoid tissue has occasionally been described, as for measles virus (White & Boyd, 1973), it is apparent that much more work in this field is needed. Further investigations to determine the effects of CMV, RSV, and other viruses on the role of the thymus in T cell maturation and development are now in progress. In addition, we are attempting to show that thymic tissue, obtained at autopsy from children who died of any of a number of immunosuppression-related disorders, may be infected by CMV, RSV, and other viruses, using a variety of immunological and molecular hybridization procedures.

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