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Susceptibility of human lymphocyte populations to infection by herpes simplex virus

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Summary. The antibody response to diphtheria toxoid by cultured tonsil cells was suppressed by herpes simplex virus during its inductive stage. Since only T lymphocytes readily supported virus replication, this immunosuppression may be attributed to a selective effect of the virus on this population of cells.

INTRODUCTION

Many viral infections interfere with immune responses (Notkins, Mergenhagen & Howard, 1970) and, in experimental animals, viruses have been shown to affect lympho-reticular cells directly (Woodruff & Woodruff, 1975). However, it is not certain that lymphocytes are similarly inactivated in human viral infections. Several viruses replicate in cultures of human mononuclear cells which have been stimulated with mitogens *in vitro* and may depress the response of these cells to such stimulation (Wheelock & Toy, 1973). In contrast little attention has been given to the effects of virus infections on human lymphoid cells that are engaged in more specific immune responses. Furthermore, the susceptibility of different lymphocyte populations to virus infections is of practical interest because of the suspicion that virus persistence in lympho-reticular cells may be related to the pathogenesis of several immunopathological diseases (Datta & Schwartz, 1976). This paper describes the suppression of antibody synthesis induced in human tonsil cells by herpes simplex virus and indicates that this agent affects the responding cells in a selective manner.

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MATERIALS AND METHODS

Immunological techniques

Antigen. Diphtheria toxoid (Wellcome Laboratories) was dialysed to remove preservatives and 1lf was added to the cultures when these were established.

Tonsil cultures (modified from Platts-Mills & Ishizaka, 1975). The cells were teased from freshly removed tonsils and washed in Hanks's BSS containing penicillin, streptomycin and nystatin. $4{\cdot}0 \times 10^7$ cells in 2 ml Eagles MEM suspension medium (Flow Laboratories) reinforced with $NaHCO_3$, 2 g/l, 18 per cent foetal calf serum, L glutamine, penicillin, streptomycin, gentamycin and nystatin were cultured in 35-mm diameter plastic petri dishes in a humid atmosphere of 5 per cent CO_2 in air. After 24 h the cells were washed three times in Hanks's BSS to remove excess antigen after which $1{\cdot}0 \times 10^7$ cells in 1·0 ml of fresh medium were transferred to modified Marbrook chambers (Marbrook 1967). These consisted of an inner chamber, containing the cultured

cells, separated by dialysis membrane from a reservoir of 20 ml RPMI 1640 medium, reinforced with $NaHCO_3$, 2 g/l, penicillin, streptomycin, nystatin and 10 per cent foetal calf serum. The cultures were maintained for a further 6 days after which the supernatant fluids were assayed for total and class specific immunoglobulin (Ig) and for specific antibody to diphtheria toxoid.

Separation and characterization of lymphocyte populations. T lymphocytes were isolated by rosette formation with neuraminidase treated sheep red cells (Bentwich, Douglas, Skutelsky & Kunkel, 1973) followed by density gradient centrifugation (Hersey, Edwards & Edwards, 1976). T lymphocytes were detected by rosette formation with sheep red cells and B lymphocytes by detecting surface Ig receptors with indirect immunofluorescence (Brown & Greeves, 1974). Macrophages were isolated by adherence to glass cover slips. Cells of different sizes were separated by a modified (Denman & Pelton, 1973) velocity sedimentation technique at 1 *g* (Miller & Phillips, 1969).

Cell viability. The criteria for cell viability were exclusion of trypan blue and morphology observed by phase-contrast microscopy.

Measurement of antibody to diphtheria toxoid. Antibody to diphtheria toxoid was measured by a double antibody radioimmunoassay. 10 μg of diphtheria toxin labelled with ^{125}I (Klinman & Taylor, 1969) was incubated for 2 h at 37° with 0·1 ml of undiluted culture supernatant and, as carrier, 0·1 ml of a 1 in 60 dilution of human serum lacking detectable antibody to diphtheria toxoid. Next, 0·1 ml of goat antibody to total or class-specific human Ig was added and the tubes were incubated for a further 16 h at 37°. The resulting precipitates were washed three times with phosphate-buffered saline and the radioactivity was counted. The amounts of antibody in the culture fluids were calculated from a standard curve constructed from the reaction between diphtheria toxoid and a strongly reactive human antiserum. Since IgG antibody greatly predominated only the results concerning this class of antibody are presented.

Measurement of Ig production. 1·0 ml of rabbit antiserum to human total Ig, IgG or IgM (Dako immunoglobulins, Copenhagen, Denmark) diluted 1/3000 with 0·06 M barbitone buffer at pH 9·55, was allowed to adsorb to polystyrene tubes, 0·8 cm in diameter (M and H Plastics), for 90 min at room temperature. After the unadsorbed contents had been discarded, the tubes were washed and 1·6 ml of a 0·5 per cent solution of bovine serum albumin was added to each tube. After incubation for a further 1 h at room temperature, the tubes were twice washed with BSS. 0·1 ml of the culture supernatant to be assayed was diluted 1/10 with BSS and the 1·0 ml was added to each tube. The tubes were again incubated for 16 h at 37°. After adding 0·1 ml of human IgG labelled with ^{125}I (Klinman & Taylor, 1969) to each tube, the tubes were incubated for a further 16 h. Finally the unbound contents were discarded and, after two further washings, the radioactivity was counted. Control experiments showed that only negligible amounts of Ig could be detected in repeatedly frozen and thawed extracts of freshly obtained tonsil cells. This indicated that the Ig measured at the end of each experiment was synthesized during the period of culture.

Virological studies

Virus strain. Herpes simplex virus type I, (HSV) HFEM strain, was grown in chick embryo fibroblasts.

Infection of cultures. The cultures were routinely infected at a multiplicity of infection (number of infectious particles/cell) of 0·1. At this level of infection, all virus is absorbed and none can be recovered in repeated washings.

Harvesting of cultures for infectivity assays. 0·25 ml aliquots consisting of cells resuspended in the culture supernatants were stored at −70° until assayed. The cells were subjected to one further cycle of freezing and thawing immediately before assay in order to release cell-associated virus. Preliminary experiments showed that viral infectivity measured in the total culture or in supernatant fluid alone did not significantly differ.

Virus assay. Virus titres were measured as plaque-forming units (pfu) on chick embryo fibroblasts by a semi-micromethod (Rager-Zisman & Merigan, 1973).

Surface staining for herpes simplex virus. $2{\cdot}0 \times 10^6$ cells were incubated for 30 min at room temperature with 0·1 ml of a 1 in 5 dilution of fluorescein-labelled

Table 1. Immunological function of tonsil cell cultures

Material synthesized	Amounts of material synthesized per culture (range)
Total IgG	18·0–41·5 μg
Total IgM	9·9–56·0 μg
Diphtheria toxoid (IgG) antibody	
(i) Immunized cultures	1·2–4·7 anti-toxin units*
(ii) Unimmunized cultures	0·2–1·6 anti-toxin units*

Cultures immunized with 1 lf diphtheria toxoid. Data for Ig synthesis were pooled from immunized and unimmunized cultures. Range refers to cultures on thirty separate tonsils.

* The term units in this case refers to arbitrary units used for this investigation, and measured as described in the text.

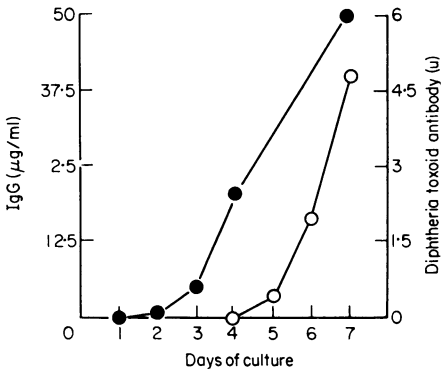

Figure 1. Synthesis of IgG antibody by cultured human tonsil cells. $1{\cdot}0 \times 10^7$ cells were stimulated with 1 lf diphtheria toxoid and the supernatant assayed for IgG and specific antibody. The kinetics of IgM production (not shown) were similar. (●) IgG synthesis; (○) antibody to diphtheria toxoid.

rabbit anti-serum to HSV type 1 (Dynatech Labs Ltd) whose specificity was checked by its failure to stain uninfected cells.

Infection of cultures. In most experiments the cells were infected after transfer to Petri dishes or to modified Marbrook chambers. However, since only small numbers of separated cells in each population were available, $0{\cdot}2 \times 10^6$ of these cells were cultured in the wells of microtitre plates. In some experiments Ig production was stimulated with pokeweed mitogen (PWM) 5 μl/well rather than diphtheria toxoid and the cultures were infected with HSV after a further 24 h.

RESULTS

Ig and antibody synthesis in tonsil cell cultures

The synthesis of both Ig and specific antibody could be detected from day 5 onwards and was maximal by the 7th day. The range of values is given in Table 1 and a representative experiment is depicted in Fig. 1. Accordingly, in order to measure the effects of virus infection, the cultures were usually harvested on day 7.

Immunosuppression by HSV

Antibody production was suppressed in cultures which were infected during the first 48 h after antigenic stimulation but thereafter the response was unaffected (Table 2). Ig synthesis was partially but less profoundly suppressed by virus infection irrespective of the duration of culture (Table 2).

Table 2. Immunosuppression by herpes simplex virus (HSV)

Age of culture when infected (h)	Per cent suppression		
	Diphtheria toxoid antibody	IgG	IgM
0	76·8 (70·6–83·2)	30·8 (20·2–36·2)	27·8 (10·0–46·5)
24	67·7 (65·7–70·6)	41·4 (40·7–43·1)	17·2 (15·4–19·0)
48	64·0 (58·8–69·2)	—	—
72–168	0 (0–4·6)	44·4 (42·2–46·6)	32·8 (20·2–53·7)

All cultures were stimulated with diphtheria toxoid, 1 lf/culture, and assayed for antibody IgG and IgM production after 7 days. HSV added at a multiplicity of infection of 0·1; per cent suppression indicates response of infected compared with uninfected cultures from the *same* tonsil. Range from assays at least in duplicate from at least eight tonsils.

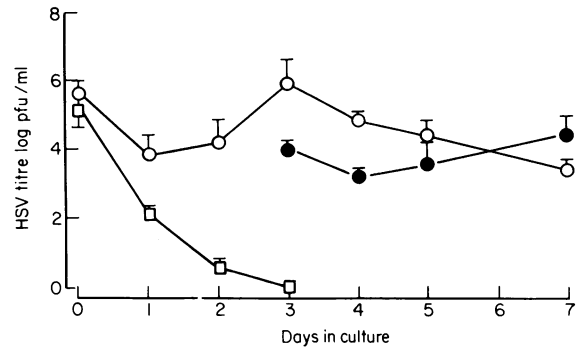

Figure 2. Replication of herpes simplex virus (HSV) in human tonsil cells. Cultures from a single tonsil were infected with HSV at a multiplicity of infection of 0·1 when the cultures were established (day 0) or 72 h later (day 3). Results are mean (+s.d.) for triplicate cultures, each assayed on three occasions. (○) infected day 0; (●) infected day 3; (□) thermal inactivation of virus (in medium alone).

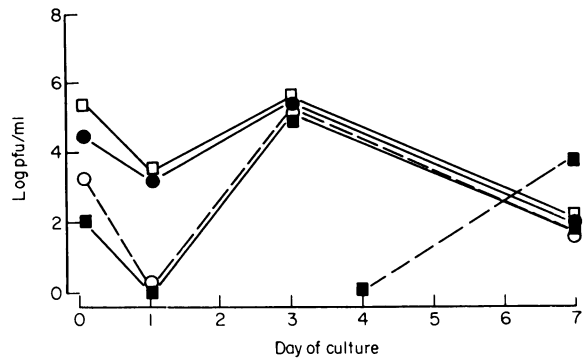

Figure 3. Limited replication of herpes simplex virus (HSV) with different multiplicities of infection. Cultures from a single tonsil were infected on day 0 with HSV at different multiplicities of infection (MOI) and the supernatants were assayed at intervals. In other tonsil cultures virus growth with an MOI of 1×10^{-1} (see Fig. 2) and 1×10^{3} (not shown) was similar to that shown for 5×10^{-3} in this figure. MOI: (□) 5×10^{-3}; (●) 5×10^{-4}; (○) 5×10^{-5}; (■—■) 5×10^{-6}; (■- - -■) 5×10^{-7}.

Viral replication in tonsil cultures

When cultures were infected at different times after these were established virus replicated to comparable titres whether HSV was added immediately after culturing or only after 72 h (Fig. 2). Furthermore the eventual titre attained was similar whatever the initial multiplicity of infection (Fig. 3). These observations indicated that the number of cells able to support the replication of HSV was limited. This conclusion was supported by immunofluorescence studies in which the number of cells giving positive immunofluorescent staining for HSV never exceeded 20 per cent (Table 3). This was regardless of the multiplicity of infection (Fig. 3). This finding could not be attributed simply to the destruction of potentially permissive cells since viable cells, although reduced in numbers compared with those in uninfected cultures, were preponderantly uninfected even at the highest initial multiplicity of infection, 10^3, that was tested. Results of such an experiment are given in Table 4.

Susceptibility of lymphocyte sub-populations to virus infection

The ability of HSV to suppress the antibody response to diphtheria toxoid during a limited period only after antigen stimulation could be explained if lymphocyte populations differ in their susceptibility to infection by this virus. Accordingly lymphocyte populations were isolated from tonsils, stimulated with PWM or diphtheria toxoid, and infected with HSV. Whereas virus replicated readily in cultures of T lymphocytes, non-rosetting cells, predominantly B lymphocytes, were consistently non-permissive (Fig. 4 and Table 5). However, the response of B lymphocytes to antigen or PWM requires the co-

Table 3. Proportion of tonsil cells infected by herpes simplex virus (HSV)

Days after infection	0	1	2	3	4	7
Immunized cultures	<0·01	3·0 (2–4)	5·0 (4–6)	13·5 (8–20)	9·0 (8–10)	11·5 (6–15)
Control cultures	<0·01	2·7 (1–4)	4·5 (3–6)	11·3 (6–16)	8·2 (8–10)	9·0 (5–12)

All cultures were infected with HSV at a multiplicity of infection of 0·1 and examined for cell-associated virus by immunofluorescence. Immunized cultures were stimulated with 1 lf diphtheria toxoid. Figures are mean (and range) of six experiments.

Table 4. Proportion of tonsil cells infected by herpes simplex virus (HSV)

Hours after infection	24		72	
	Infected	Uninfected	Infected	Uninfected
If pos. (%)	15·0	<0·1	20·0	<0·1
Cells/culture $\times 10^{-6}$	3·4	4·8	3·2	4·0
Viability (%)	60·0	80·0	70·0	90·0

$10{\cdot}0 \times 10^6$ tonsil cells were stimulated with 1 lf diphtheria toxoid. Infected = Cultures infected with HSV at a multiplicity of infection of 0·1. lf pos = Cells with positive immunofluorescence staining for HSV.

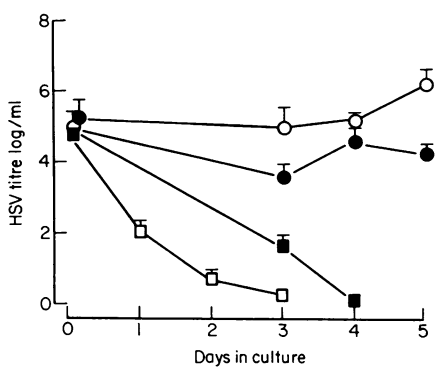

Figure 4. Susceptibility of tonsil lymphocyte sub-populations to infection by herpes simplex virus (type 1). T lymphocytes and B lymphocytes were infected separately with HSV at a multiplicity of infection of 0·1. Virus growth in these cells was compared with that in unseparated cells from the same tonsil and that in medium alone (thermal inactivation). Figures are mean (+1 s.d.) of three assays on triplicate cultures. (○) T lymphocytes; (■) B lymphocytes; (●) unseparated cells; (□) thermal inactivation.

Table 5. Composition of tonsil cell preparations

Preparation	SRFC (%)	Ig pos (%)
Unseparated	26·5 (25–30)	67·2 (65–70)
Rosetting cells	83·9 (80–85)	7·5 (5–10)
Non-rosetting cells	0·5 (0–1)	84·3 (70–95)

Figures are mean (and range) of four experiments on separate tonsils. Cells forming rosettes with sheep RBC were separated by density sedimentation and the different populations were assayed for rosette forming cells (SRFC), and for membrane Ig by immunofluorescence (Ig pos).

operation of T lymphocytes or their product (Geha, Schneeberger, Rosen & Merler, 1973; De la Concha, Oldham, Webster, Asherson & Platts-Mills, 1976). It could thus be argued that these cells would not in isolation undergo the metabolic changes necessary for virus replication (Wheelock & Toy, 1973). Therefore, in further experiments, cultures of unseparated tonsil cells were stimulated for an initial period of 24–72 h with PWM or diphtheria toxoid after which

B lymphocytes were isolated and then exposed to HSV. Similarly tonsil cells, deprived of T lymphocytes, were grown in medium harvested from 24–72 h old cultures of PWM stimulated, unseparated tonsil cells. However, despite all these manoeuvres, no replication of HSV in B lymphocytes was achieved.

The decline in virus infectivity was similar in cultures of glass-adherent macrophages and of B lymphocytes from which macrophages had been removed. Similar results were obtained in cultures of macrophages which had been non-specifically activated (Zisman & Denman, 1973).

Populations of tonsil cells of different size, which were separated by velocity sedimentation, all supported virus replication to comparable extent.

DISCUSSION

There is considerable information about the mechanisms of viral immunosuppression in various experimental systems and, clearly, some viruses affect immune responses in a highly selective manner. Thus, some oncornaviruses depress antibody synthesis by their effect on lymphocytes only at an early stage in the maturation of the response (Cerny, McAlack, Ceglowski & Friedman, 1971; Dracott, Wedderburn & Salaman, 1972). There are also indications that the ability of viruses to replicate in human lymphoid cells is subject to similar restrictions. Thus B but not T lymphocytes possess receptors for EB virus (Greaves, Brown & Rickinson, 1975) probably through their Fc receptors (Yefenof, Klein, Jondal & Oldstone, 1976). In our experiments herpes simplex virus type I (HSV) replicated readily in T lymphocytes, but B lymphocytes appeared entirely non-permissive for this agent. Furthermore antibody synthesis to diphtheria toxoid was suppressed only during the initiation of the response. These findings suggest that HSV interfered with the helper function of T lymphocytes. Similarly measles virus depresses the anti-hapten antibody response in mice by its action on this population (McFarland, 1974). Although less markedly affected, Ig synthesis was also depressed, presumably because HSV also interfered with T lymphocytes needed to initiate immune responses *in vitro* to a variety of microbial and other antigens.

The possible immunosuppressive effects of interferon must also be considered (Johnson, Smith & Baron, 1975) since both antigenic stimulation and viral infection would be expected to stimulate its production. Moreover human tonsil cells behave like other lymphoid cells in this respect (Marchenko, Poblotsky & Krivokhats Kaya, 1976). However, the relatively low titres of interferon that are detected cannot be correlated with the selective immunosuppression that we have described either in terms of timing or extent (Zisman & Denman, 1973). In addition, cultures both freshly established and several days old supported the replication of HSV equally well even though interferon was secreted throughout this period and this virus is sensitive to its action (Rasmussen & Farley, 1975). Nevertheless the contribution of interferon is not entirely excluded; not only may interferon produced by lymphoid cells possess special characteristics (Valle, Jordan, Haahr & Merigan, 1975) but the immunosuppressive properties of interferon can be distinguished from its effect on virus replication (Dahl & Degre, 1975) and therefore might not have inhibited the growth of HSV in these cultures.

Judged by immunofluorescence the maximal proportion of infected cells appeared never to exceed 20 per cent but this method may have overestimated the true number since it does not distinguish between cells replicating virus and non-producing cells to which virus has absorbed. On the other hand neither this technique nor infectious centre assays enumerate cells infected with incomplete, non-infective virus particles. Nevertheless the number of infected cells did not exceed even that of the T lymphocytes in the cultures. Several factors may have limited the extent of viral growth which was similar irrespective of the initial multiplicity of infection. Thus cultured cells show a cyclical resistance and susceptibility to virus infection which is not determined by obvious immunological factors (Hotchin, 1973). Furthermore, the susceptibility of lymphoid cells to herpes virus infection could theoretically be determined by variations in cell cycle. However, populations isolated on the basis of cell size, a procedure which synchronizes cell populations in addition to separating those with functional differences (Miller & Phillips, 1969) were equally susceptible.

Some viruses, such as adenovirus in tonsils (Israel, 1962), persist in human lymphoid cells for prolonged periods without obvious pathological consequences. However there are several immunopathological disorders of man and experimental animals resulting from virus infections in which such cells constitute a continuous reservoir of virus infection. Furthermore

not only is the specific response to the infecting agent often impaired (Wheelock & Toy, 1973) but also many viruses induce a variety of other aberrations including disturbances of tolerance thereby, in theory, leading to auto-immunity (Datta & Schwartz, 1976). The preferential sequestration of viruses in T lymphocytes may confer some advantages on the host since efficient antigen processing would thereby be combined with viral inactivation (Zisman & Denman, 1973). Thus the transient depression of circulating T lymphocytes (Scheinberg, Blacklow, Goldstein, Parrino, Rose & Cathcart, 1976) with viral antigen detectable in these cells (Wilson, Planterose, Nagington, Park, Barry & Coombs, 1976) may reflect the operation of this defence mechanism. The ability of herpes viruses other than HSV, such as cytomegalovirus (Olding, Jensen & Oldstone, 1975) and EB virus (Yefenof *et al.*, 1976) to replicate in B lymphocytes may enable these strains to evade this form of host defence.

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