Fate of herpes simplex virus in lymphocytes from inflammatory joint effusions. II. Mechanisms of non-permissiveness

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SUMMARY Mononuclear cells which were isolated from rheumatoid synovial effusions and cultured without prior separation failed to support the growth of herpes simplex virus (HSV). However, subpopulations of T lymphocytes separated on the basis of cell size or receptors for sheep red cells were, in isolation, permissive for this virus. Virus growth in these permissive cells was blocked by cocultivation with the remaining mononuclear cell populations.

A companion paper describes experiments in which mononuclear cells isolated from rheumatoid synovial effusions failed to support the growth of herpes simplex virus type 1 (HSV) (Appleford and Denman, 1979). A number of possible explanations for this finding were excluded such as the blocking of virus receptors on lymphocytes or the lack of appropriately activated lymphocytes able to replicate HSV. In this paper we describe the results of further experiments which indicate that rheumatoid synovial effusions contain lymphocytes which, in isolation, can support the growth of HSV but lose this ability when cultured in combination with the remaining mononuclear cell populations.

Materials and methods

CLINICAL MATERIAL

Blood samples and synovial effusions from the knee joints were obtained from patients with inflammatory arthritis, as previously described (Appleford and Denman, 1979).

IMMUNOLOGICAL METHODS

Isolation of mononuclear cells. After treating the joint fluids with hyaluronidase, mononuclear cells from both joint fluids and blood were isolated by density gradient sedimentation on Ficoll/Triosil (Boyum, 1968), as described in the previous paper (Appleford and Denman, 1979).

Cell separation methods. Separation by cell size was carried out by a modification (Denman and

Pelton, 1973) of the unit gravity sedimentation technique (Miller and Phillips, 1968). The conditions of separation by this method were standardised. In each experiment a constant volume of 1/3 to 2/3 of a 20% Ficoll gradient was used to sediment cells for a period of 2½ hours at room temperature. At the end of the sedimentation period 25 ml fractions were collected.

Macrophages were isolated by adherence to glass or plastic surfaces (Mosier, 1967). T lymphocytes were separated by rosette formation with neuraminidase-treated sheep red blood cells (SRBC) (Weiner *et al.*, 1973) followed by density sedimentation on Ficoll/Triosil (Hersey *et al.*, 1976).

Mitogen stimulation. Suspensions of mononuclear cells at 10^6 cells/ml were stimulated with phytohaemagglutinin (PHA, Wellcome purified grade) at $0.8 \mu\text{g}/10^6$ cells. Stimulation was assessed by ^{14}C -thymidine uptake over an 18-hour period 48 hours after stimulation (Janossy and Greaves, 1977).

Coulter counting and cell sizing. Coulter counter model ZB1, standardised for counting human mononuclear cells was used. A P64 size analyser with X-Y print-out attachment gave plots of size distribution on a scale standardised by analysis of pollen grains in the mononuclear cell size range.

Differential counts. These were performed on cytocentrifuge preparations stained with May-Grunwald-Giesma.

Cell viability. This was measured by 0.1% trypan blue exclusion.

Lymphocyte subpopulations. B cells with surface immunoglobulin were identified by direct immunofluorescence using a fluorescein-labelled sheep

antibody to total human immunoglobulin (Hoechst Ltd.). T lymphocytes were assayed by rosetting with neuraminidase-treated sheep red blood cells (Weiner *et al.*, 1973). Macrophages were identified by phagocytosis of yeast particles using phase contrast microscopy, and by acridine orange staining.

VIROLOGICAL METHODS

Methods for the preparation of virus stocks, infection of mononuclear cells, and measurement of virus titres were the same as those detailed in the accompanying paper (Appleford and Denman, 1979.)

Results

SEPARATION OF EFFUSION LYMPHOCYTES BY SIZE DIFFERENCE

Mononuclear cells isolated from the synovial effusion and blood of patients with rheumatoid arthritis were separated by velocity sedimentation at 1 g. The cell fractions were amalgamated into 3 pools termed 'small', 'medium', and 'large' on the basis of their sedimentation rate and hence their cell volume. A comparison of the sedimentation profiles of nucleated cells from blood and effusions of the same donors (Fig. 1, Table 1) showed that effusions often contained a distinctive peak of cells in the large cell range, which was not present in peripheral blood. The sedimentation profiles of blood lymphocytes obtained from patients with rheumatoid arthritis and from normal donors were identical.

Characterisation of the cells isolated in each cell pool indicated that the 'large' pool contained cells in the lymphoblast size range as well as macrophages; in addition, the 'large' pool from the effusion contained those granulocytes not removed

Fig. 1 Unit gravity sedimentation of blood and effusion lymphocytes from a patient with rheumatoid arthritis. Cells were sedimented on a 1/3–2% Ficoll gradient for 2½ hours, after which 25 ml fractions were collected and analysed.

by Ficoll/Triosil purification. The 'small' cell pool from both effusion and blood was relatively enriched for T lymphocytes, whereas the 'large' pool from the same source were relatively depleted of such lymphocytes.

All pools of blood lymphocytes supported the growth of HSV although large cells were less efficient in this respect (Figs. 2 and 3). However, the fate of HSV was different in lymphocyte populations of comparable size separated from synovial effusions.

Two patterns of results were obtained. In 4 patients none of the lymphocyte pools supported viral growth. In contrast, both small and medium sized lymphocytes from the synovial effusions of 2 patients

Table 1 Characterisation of mononuclear cell pools from blood and synovial effusions of patients with rheumatoid arthritis separated by velocity sedimentation

Cell pool ¹		Sedimentation rate (mm/h)	Size range (μm ³)	% Of cells recovered	% Lymphocytes	% Macrophages	% Sm Ig +ve	% E Rosette +ve
Large	Effusion	7.18–16.0	642.0–2143.0	44.16 ³	23.84	11.7	5.7	17.7
	Blood			(28.2–57.8)	(4.0–34.0)	(2.0–32.0)	(3.4–9.0)	(9.0–24.1)
Medium	Effusion	5.0–7.18	374.0–642.0	30.65	82.25	4.97	3.42	33.02
	Blood			(20.6–45.1)	(77.0–85.0)	(2.0–8.9)		
Small	Effusion	3.3–5.0	200.0–374.0	39.78	47.24	5.1	1.76	29.33
	Blood			(22.2–50.0)	(35.0–62.0)	(2.1–13.3)	(1.3–2.0)	(21.0–34.0)
Unseparated	Effusion	—	—	41.32	92.5	1.01	2.32	62.4
	Blood			(25.8–54.6)	(90.0–100.0)	(0.4–3)		(52.0–71.0)
	Effusion	—	—	16.09	76.95	0.4	3.63	64.5
	Blood			(9.0–21.79)	(57.0–91.0)	(0.1–2)	(0.6–0)	(63.0–66.0)
	Effusion	—	—	27.85	98.75	0.25	7.12	82.10
	Blood			(25.0–30.0)	(95.1–100)	(0.1–0)		(78.5–87.0)
	Effusion	—	—	59.54	6.0	8.34	56.86	56.86
	Blood			—	(32.5–87.0)	(3.0–12.5)	(5.0–14.0)	(32.6–75.0)
	Effusion	—	—	92.0	2.85	16.6	61.45	61.45
	Blood			—	(90.0–93.0)	(2.0–4.0)	(13.4–21.0)	(60.9–62.5)

¹ Analysis of 4 blood samples and 6 synovial effusions. ² Data from 1 observation only. ³ Figures are the mean (and range). Large, medium, and small cells collected as in Fig. 1.

Fig. 2 Growth of HSV in blood subpopulations from a patient with rheumatoid arthritis, separated by velocity sedimentation. ●—● unseparated cells, ○—○ large cells, □—□ medium cells, △—△ small cells. 40×10^6 cells sedimented as described in Fig. 1 and collected in 3 pools were stimulated with PHA and infected with HSV at an MOI of 0.1. The size range of the cells in each pool is given in Table 1.

Fig. 3 Fate of HSV in lymphocyte subpopulations separated by velocity sedimentation. U=unseparated cells; L=large cells; M=medium cells; S=small cells. HSV titres are the highest observed after 3–5 days of culture. The horizontal bar shows the mean virus titres.

supported virus growth as indicated in Fig. 3 and illustrated in more detail in Fig. 4.

In addition 5 rheumatoid synovial effusions were separated by velocity sedimentation to give 4 pools of cells with a narrower range of cell size in each pool. All cell pools from 3 effusions failed to support viral growth, but virus grew to titres of $2.5\text{--}3.6$ logs in cells of less than $750 \mu\text{m}^3$ isolated from the remaining 2 effusions.

SEPARATION OF EFFUSION LYMPHOCYTES BY SURFACE MARKERS

The preceding separation experiments suggested that synovial effusions contain cells potentially able to support HSV replication. When tonsil cells or normal blood lymphocytes are exposed to HSV this virus grows exclusively in a limited number of T lymphocytes (Pelton *et al.*, 1977). Similarly, when blood lymphocytes from 10 patients with rheumatoid arthritis were infected with HSV only T lymphocytes supported virus growth, giving titres comparable with those obtained in cultures of normal blood T lymphocytes (Fig. 5). Since the slowly sedimenting cells obtained by velocity sedimentation of synovial effusions were greatly enriched for T lymphocytes it seemed likely that these were the

Fig. 4 Growth pattern of HSV in lymphocyte subpopulations from a rheumatoid synovial effusion. ●—● unseparated cells, ○—○ large cells, □—□ medium cells, △—△ small cells. Lymphocyte pools obtained by velocity sedimentation correspond to those illustrated in Fig. 1. Cultures were stimulated with PHA and infected with HSV as described in legend to Fig. 2.

cells which supported viral growth. Indeed, T lymphocytes isolated from all 8 synovial effusions tested were permissive for HSV growth. The titres were comparable with those obtained in cultures of peripheral blood T lymphocytes from both normal donors and those with rheumatoid arthritis (Fig. 5). Details of a representative experiment are given in Fig. 6.

These results suggested that rheumatoid synovial effusions contain T lymphocytes able to support the growth of HSV but that this potential growth is suppressed by other mononuclear cells. To test this hypothesis T lymphocytes from synovial effusions were cultured in combination with other lymphocytes from the same source at varying ratios. As a control T lymphocytes were cultured with comparable ratios of T lymphocytes that had been treated with actinomycin D to block viral growth or with non-

Fig. 5 Growth of HSV in T lymphocytes isolated from rheumatoid peripheral blood and synovial effusions. U=unseparated cells, T=lymphocytes. T lymphocytes were isolated from the peripheral blood of normal controls and from the blood and effusions of patients with rheumatoid arthritis (RA). Conditions of culture and HSV infection as in Fig. 2. Virus titres are maximal observed after 3–5 days culture. (T cells constituted $69.02 \pm 14.5\%$ of the total effusion lymphocytes).

Fig. 6 Growth of HSV in T lymphocytes. ●—● blood T lymphocytes, ■—■ effusion T lymphocytes, ○—○ blood unseparated cells, □—□ effusion unseparated cells. T lymphocytes were isolated from the effusion infected with HSV under the conditions described in Fig. 2. Figures are the mean ($\pm$ ISD) of virus titration of triplicate cultures.

T lymphocytes from autologous blood. The results are summarised in Table 2 and illustrated in Fig. 7.

In 3 experiments T lymphocytes from synovial effusions cultured at a ratio of 40% untreated to 60% actinomycin-D-treated cells or 25% effusion T cells to 75% autologous blood non-T cells still supported viral growth. This was in keeping with our earlier observations (Appleford and Denman, 1979) in which permissive normal blood lymphocytes still supported HSV replication provided that the non-permissive cells with which the permissive cells were mixed did not constitute more than 70% of the total number. Non-T lymphocytes from synovial effusions behaved differently from other non-permissive populations. In 5 experiments non-T effusion cells inhibited viral growth in effusion T cells even when the permissive T lymphocytes made up more than 60% of the total number of cultured cells. In 4 out of 5 experiments synovial effusion non-T cells inhibited viral growth only in T lymphocytes from the same source and not in T lymphocytes isolated from the blood of the same patient. In the fifth experiment the effusion cells also prevented the growth of HSV in autologous blood T lymphocytes (Table 2).

Table 2 Inhibition of HSV growth in T lymphocytes by other lymphocyte subpopulations

Non-T	% Effusion T lymphocytes				% Blood T lymphocytes			
	25	50	75	100	25	50	75	100
Effusion ¹	1.02 (5) ³ (0-1.9)	0.64 (5) (0-1.9)	0.92 (5) (0-2.3)	5.0 (5) (3.8-5.6)	4.2 (2) (3.4-5.0)	5.05 (4) (4.8-5.4)	4.85 (2) (4.8-5.2)	5.12 (4) (4.1-5.5)
Blood	4.5 (3) (2.8-5.4)	3.7 (3) (2.1-4.6)	4.8 (3) (3.8-5.4)	5.4 (3) (5.2-5.6)	5.1 (2) (4.8-5.4)	4.9 (2) (4.6-5.2)	4.7 (1)	5.1 (2) (4.8-5.4)
Actinomycin-D	2.8 (3) (1.9-4.2)	4.06 (3) (3.8-4.3)	4.5 (3) (4.2-4.9)	5.3 (3) (5.1-5.6)	3.5 (2) (3.1-3.9)	4.4 (2)	—	5.4 (2) (5.3-5.5)
Treated T								

¹ Non-T lymphocytes mixed with T cells in concentrations to make 100%. ² Virus titre is the maximum between days 3 and 5 of culture. ³ Figures are the mean virus titre in log plaque forming units (PFU)/ml (number of experiments) and (range). ⁴ Non-permissive effusion non-T:blood T lymphocyte mixture illustrated separately.

Fig. 7 Inhibition of HSV growth in T lymphocytes by other lymphocyte populations. $\blacktriangle$ - $\blacktriangle$ effusion T lymphocytes; $\blacksquare$ - $\blacksquare$ effusion non-T lymphocytes; $\square$ - $\square$ blood T lymphocytes; $\circ$ - $\circ$ effusion T lymphocytes; actinomycin-D-treated T lymphocytes. T lymphocytes isolated from a rheumatoid blood and synovial effusion were cultured with varying proportions of non-T lymphocytes from the blood and effusion of the same patient. Conditions of infections in Fig. 2. Figures are mean ($\pm$ ISD).

The non-T lymphocytes responsible for blocking viral growth have not been characterised. However, in 2 experiments the inhibitory effect of effusion non-T lymphocytes on effusion T cells was not ablated by removal of glass-adherent cells.

Discussion

A previous paper (Appleford and Denman, 1979) reported that lymphocytes from the effusions of

patients with several forms of inflammatory arthritis failed to support the growth of HSV. Moreover, it was shown that this non-permissiveness was not caused by blockade of lymphocyte virus receptors, defective lymphocyte function, or the effects of residence in the inflammatory effusion.

Our experiments show that there is indeed a subpopulation of lymphocytes in these effusions which in isolation supports the growth of HSV. Judged by velocity sedimentation this population consists of small and medium sized lymphocytes and was detected in 4 out of 11 effusions examined. In all 4 effusions the permissive population was enriched for T lymphocytes and depleted of those cell types which constitute the majority of the large cell fractions isolated from both blood and synovial effusions. Whereas the large cell fractions isolated from blood also supported HSV replication, similar fractions from synovial effusions were entirely non-permissive.

T lymphocytes isolated from synovial effusions supported the growth of HSV to titres comparable to those obtained in T lymphocyte cultures from the blood of normal donors, indicating that the failure of virus to grow in effusion lymphocytes was not due to the deletion of the susceptible subpopulation. Furthermore, the percentage of T cells in the effusions was the same and in some cases higher than that in cultures of autologous blood lymphocytes which did support virus growth. Indeed HSV still failed to grow in small and medium cell pools from 7 effusions whose T-lymphocytes content exceeded 63%, indicating that further purification was still necessary for these cells to support virus growth. These results suggested that the growth of HSV in T lymphocytes was inhibited by a subpopulation of cells separating in the non-T cell fraction. The mixing experiments in which effusion T lymphocytes were diluted with varying ratios of non-T lymphocytes from the same source confirmed this conclusion. It should be emphasised that similar mixtures of lymphocyte subpopulations from normal blood did not inhibit virus growth. Further experiments

indicated the inhibiting cells were not macrophages or T lymphocytes and were enriched in the rapidly sedimenting fraction, but these have not yet been further characterised.

The inhibition of virus growth could be accounted for in several ways. Some lymphocytes in the effusion might have been cytotoxic for virus-infected T lymphocytes and could have killed these cells before virus replicated (Zinkernagel and Althage, 1977). As only a small percentage of human T lymphocytes are infected initially by HSV (Pelton *et al.*, 1977) this killing would not result in obvious reduction in viable cell numbers. This effect is unlikely to be mediated by classical interferon, since vesicular stomatitis virus (VSV) replicated in the effusion cell cultures and infection by this virus is extremely sensitive to interferon. Moreover, interferon could not be detected in the non-permissive cultures (Appleford, unpublished observations).

Nevertheless other antiviral factors produced by lymphocytes, perhaps of a specific nature, could have been responsible (Rouse and Babiuk, 1975). It is possible that the B lymphocytes of patients with rheumatoid arthritis carry EB virus, since the sera of these patients and not normal controls contain an antibody which reacts with a characteristic nuclear antigen in B lymphocytes infected by this virus (Alspaugh *et al.*, 1978). This infection in B lymphocytes could induce resistance in T lymphocytes to infection by other herpes viruses.

This would imply that the effusions contain lymphocyte populations participating in an antiviral response which can be detected by the failure of HSV to grow in T lymphocytes. There is indeed evidence for antiviral resistance of this nature. Thus human lymphocytes which normally support the growth of 17D yellow fever virus lose this permissiveness if the donor has recently been immunised against the virus (Wheelock and Edelman, 1969); the non-permissiveness does not result from direct infection of the lymphocytes.

Thus this result is consistent with the hypothesis that the cells at the site of rheumatoid inflammation are engaged in an antiviral response. Although the failure of HSV to replicate in T lymphocytes from synovial effusions is unlikely to reflect interference by defective virus in these cells, the possibility

remains that other lymphocyte subpopulations harbour a persistent infection.

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