**In vitro Stimulation of Rabbit T Lymphocytes by Cells Expressing Herpes Simplex Antigens**

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**SUMMARY**

Lymphocyte stimulation responses to herpes antigens were studied using virus-infected X-irradiated cells. Rabbits were immunized with herpes simplex virus type 1 (strain HFEM) grown in RK13 cells. For *in vitro* stimulation assay BHK21 cells were X-irradiated (15,000 rad) and infected with a high m.o.i. of a temperature-sensitive (ts) mutant (N102) of HFEM strain at the non-permissive temperature (38.5 °C) of virus. Virus antigens were expressed on the infected cells and there was no leakage of infectious virus into the medium at 38.5 °C. T lymphocytes from rabbits immunized with herpes simplex virus were specifically activated by herpesvirus-infected X-irradiated cells; lymph node cells from rabbits immunized with RK13 cells and from non-immune rabbits showed no proliferative response.

There are several reports of *in vitro* stimulation of rabbit lymphocytes by herpes simplex virus (HSV) antigens (Rosenberg et al., 1972; Jacobs et al., 1976; Kapoor & Nash, 1979). The stimulatory capacity of HSV preparations has been shown to be markedly dependent on the way that the antigen is prepared (Kapoor et al., 1978). When crude ultrasonicated preparations or cell membrane-associated antigens have been used for stimulation, the possible importance of association with major histocompatibility complex (MHC) antigens has generally not been ascertained. The considerable body of work demonstrating dual recognition of virus and MHC antigens in T cell recognition and effector cell reactions has recently been reviewed by Doherty (1980). Here we describe an investigation of the stimulation of rabbit T cells by herpes antigens using live virus-infected cells. Herpes-specific T lymphocyte stimulation was demonstrated but, in contrast to another report (Moorhead, 1978), it did not appear to require the help of MHC antigens.

The HSV type 1 strain HFEM was used to immunize rabbits. It was grown in RK13 cells in medium supplemented with normal rabbit serum. A temperature-sensitive (ts) mutant of this strain (N102) was used in a stimulation assay. This mutant grows readily in BHK21 cells at the permissive temperature (32 °C) but at the non-permissive temperature (38.5 °C) fails to produce infective progeny but does produce surface antigens. The mutant ts N102 is also defective in DNA synthesis (DNA-negative) at 38.5 °C.

For stimulation assay, rabbits were immunized using formalized virus (10⁶ p.f.u.) followed by intradermal injections consisting of live virus (10⁵ and 10⁶ p.f.u. respectively) at weekly intervals (Wildy, 1954). Control rabbits were immunized with RK13 cells only.

Virus infected X-irradiated BHK21 cells were used for *in vitro* virus-specific stimulation of lymphocytes. BHK21 cells (2 × 10⁸) were X-irradiated (15,000 rad) and left in a 500 ml plastic bottle in 40 ml Eagle’s medium supplemented with tryptose-phosphate broth and calf serum-ETC (X-irradiation was done to eliminate DNA synthesis by cells; it was already known that the cells took up very little [³H]thymidine 7 days after X-irradiation and approx. 60% of the cells were viable during this period as detected by trypan blue exclusion). After 2 days, the medium was decanted and live adherent cells were removed by trypsin and versene, washed, and 10⁶ cells placed in a plastic dish 5 cm in diam. in 5 ml medium in 10% CO₂ in
Fig. 1. Schematic representation of plastic dish, successively coated with (a) rabbit IgG (Y), (b) staphylococcal protein A (●) and (c) guinea-pig anti-rabbit thymocyte antibody (Y).

air, along with a coverslip to allow adhesion of X-irradiated cells to occur. Twelve h later, the cells were infected with high m.o.i. (approx. 70) of ts N102 in 1 ml medium at 38.5 °C for 1 h with intermittent shaking followed by addition of 4 ml medium and incubation for another 5 h. About 90% of cells expressed virus-specific antigens on their surface 6 h after infection at 38.5 °C as judged by an indirect immunofluorescence test (Geder & Skinner, 1971). Heat-inactivated infected cell extract was prepared by the method described earlier (Kapoor et al., 1978).

In vitro lymphocyte stimulation was studied using a microculture technique (Kapoor et al., 1978). Each culture contained $2 \times 10^5$ lymph node cells in 0.1 ml Eagle's medium supplemented with 5% foetal calf serum (FCS) and 2-mercaptoethanol (60 µM) in an atmosphere of 5% CO₂ in air. The cultures were set up in quadruplicate. Lymph node cells were obtained from rabbits immunized with HSV strain HFEM grown in RK13 cells and with RK13 cells alone, and also from non-immunized rabbits. The mutant ts N102-infected X-irradiated BHK21 cells ($5 \times 10^4$) expressing virus-specific antigens on their surface membranes were added to each culture for studying herpes-specific stimulation responses. Heat-inactivated, virus-infected, X-irradiated BHK21 cell extracts having $10^6$ p.f.u./culture (this concentration of antigen preparation has always given optimal stimulatory response; Kapoor et al., 1978) were also added in parallel cultures. Uninfected X-irradiated BHK21 cells and BHK21 cell extract were always used as a control. The cultures were incubated at 38.5 °C and harvested after 5 days using a Skatron multiwell cell culture harvester as described previously (Kapoor et al., 1978).

T lymphocytes were separated on dishes successively coated with IgG–staphylococcal protein A and guinea-pig anti-rabbit thymocyte antibody (Nash, 1976). Rabbit IgG (200 µg) was allowed to react with 1 mg carbodiimide in 2 ml saline for 30 min on 30 mm diam. Falcon Petri dishes, followed by washing and treatment with staphylococcal protein A. Millipore-filtered culture supernatant of *Staphylococcus aureus* Cowan I strain was used as a source of protein A (Arvidson et al., 1971). The Petri dish was washed and treated with guinea-pig anti-rabbit thymocyte serum (Fig. 1). The coated surface was again washed before the addition of lymph node cells. Subsequently, the dish was centrifuged at 350 g for 3 min followed by carefully filling the dish with the medium (RPMI 1640, HEPES, 2% IFCS) to form a convex meniscus on a horizontal surface. The lid was replaced avoiding formation of
Fig. 2. Virus-specific stimulation of immune effector cells by herpes antigens. Experiments (a) and (b) show relative superiority of virus-infected cells in in vitro lymphoproliferative responses using unseparated (starter) lymph node cells. 1, Antigen on intact cell surface; 2, inactivated ultrasonicated antigen. Experiments (c) and (d) show results of virus-specific stimulation of T cells by herpes antigens on infected, intact cell surfaces. 1, Using starter lymph node cells; 2, using T lymphocytes. □, Virus-immune lymph node cells; △, RK13-immune lymph node cells; ■, non-immune lymph node cells.

Air bubbles. The dish was inverted and allowed to stand for 1 h. The coated dish surface was then lifted out of the cover to leave unattached cells in the lid. Later, adherent cells were removed with jets of medium. Purity of separated cells was always checked by the mixed antiglobulin (MAG) rosette test (Hallberg et al., 1974) to confirm that the adherent cells (T lymphocytes) did not form Ig-anti-Ig rosettes.

Fig. 2 describes results of four experiments. Each experiment was done with lymph node cells obtained from different rabbits. Fig. 2(a, b) shows the results of immunospecific stimulation of unseparated (starter) lymph node cells. Lymphocyte stimulation by virus-infected X-irradiated cells was repeatedly found to be superior when compared with stimulation produced by heat-inactivated virus preparations (Fig. 2a, b). Uninfected BHK21 cell extracts did not stimulate immune and non-immune lymph node cells. Thereafter, virus-infected cells were used for the stimulation of T lymphocytes. Fig. 2(c, d) shows the results of in vitro stimulation of T cells by virus-infected X-irradiated cells. Unseparated (starter) lymph node cells and T lymphocytes (T-positive cells) were obtained from unimmunized rabbits and from rabbits immunized with strain HFEM grown in RK13 cells or with RK13 cells alone. The lymph node cells were stimulated with surface antigens on ts N102-infected live X-irradiated BHK21 cells. The results of proliferative assay revealed that both the unseparated population and the T cells from herpes type 1-immunized rabbits were immunospecifically stimulated by virus antigens. Control lymph node cells showed no stimulation. Uninfected X-irradiated BHK21 cells did not stimulate immune or non-immune lymph node cells. At the end of the culture period, the parallel cultures were checked for
leakage of infectious virus into the medium by plaque assay at 32 °C (permissive temperature of \(ts\) N102 mutant virus); none was observed. The results of MAG rosettes with separated lymph node cells revealed that the T-positive subpopulation did not form rosettes (<1 in 100 MAG-positive lymph node cells) and the non-adherent (T-negative) subpopulation had approx. 86% Ig-positive lymphocytes. Antigen-specific stimulatory responses with starter populations in experiments (c) and (d) were lower when compared with experiments (a) and (b) because lymph node cells had to stand for approx. 3 h (time required for separation of T cells) before setting up cultures.

There is much evidence that, in mice, the ability of virus-specific cytotoxic T lymphocytes to lyse virus-infected target cells is restricted by gene products encoded in the H-2K and H-2D region of the major histocompatibility complex (Doherty, 1980; Zinkernagel, 1978). Similar conclusions have been reached from \textit{in vitro} studies of virus-specific cytotoxic reactions with human lymphocytes (Sethi \textit{et al.}, 1980). It has also been shown that mixed vesicles containing virus structural proteins and H-2 antigens can trigger secondary antiviral effector cells \textit{in vitro} (Ciavarra \textit{et al.}, 1980). It is probable that MHC and virus antigens which are physically associated in the cell surface are recognized.

Most recent work has been concerned with the specificity of the cytotoxic reaction and not with the specificity of mitotic activation which is the subject of the present study. There is, however, evidence for an involvement of MHC products in immunogenicity and in antigen-induced murine T cell proliferative responses \textit{in vitro} (Miller, 1978). Yet it is clear from the results reported here that this is not invariably the case. Under the \textit{in vitro} conditions used, rabbit T lymphocytes appeared to recognize and respond to membrane-associated virus antigens expressed on a cell bearing foreign MHC antigens. Other possible interpretations of these results could be that live virus-infected BHK21 cells might have simply provided a lipid bilayer for antigen presentation, or virus antigens were being shed from the infected cells and subsequently being processed by antigen-presenting cells which then served to stimulate T lymphocytes.

We recommend the adoption of devices such as the use of \(ts\) mutants of virus in \textit{X-irradiated} target cells to those who wish to follow the lymphoproliferative responses in \textit{in vitro} systems involving virus-infected cells.

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