INFECTION AND IMMUNITY, Nov. 1974, p. 1034-1039
Vol. 10, No. 5

Plaque Size Reduction as a Measure of Viral Cell-Mediated Immunity

RICHARD L. SIMMONS, YSOLINA CENTIFANTO, AND HERBERT E. KAUFMAN
Department of Ophthalmology, College of Medicine, University of Florida, Gainesville, Florida 32610

Received for publication 31 May 1974

This new assay of viral cell-mediated immunity is sensitive, reproducible, and in many ways resembles the in vivo state. The spread of herpes simplex virus between adjacent monolayer cells was inhibited in the presence of spleen cells from guinea pigs sensitized to that virus. This in vitro control of viral growth was quantified by determining plaque size in monolayers to which were added sensitized spleen cells as opposed to nonsensitized or no spleen cells. The simple measurement of plaque size reduction as an in vitro test of viral cell-mediated immunity is described. In addition to correlating highly with skin testing and macrophage migration inhibition as a test of viral cell-mediated immunity, the ability of sensitized spleen cells to reduce plaque size developed by day 7, paralleling the onset of delayed cutaneous hypersensitivity. The specificity of this lymphocyte-mediated interaction was demonstrated by the inability of herpes simplex virus-sensitized spleen cells to alter the growth of vaccinia virus in cell culture. A ratio of sensitized spleen cells to monolayer cells of 6:1 resulted in significant plaque size reduction on both HEp-2 and conjunctiva monolayers. The data presented demonstrate the potential usefulness of plaque size reduction as a technically simple, specific, and more direct measure of cellular antiviral activity.

The increasing appreciation of viral cell-mediated immunity (CMI), especially in regard to pox-, herpes-, and papovaviruses (1), has led to a new importance being attached to the in vitro measurement of cellular immunity in virus infection. This interest is understandable in view of the frequency of opportunistic intracellular infection leading to death in the immunosuppressed host, the debility and despair accompanying recurrent viral disease in the otherwise immunocompetent host, and the viral role in the pathogenesis of autoimmune disease and the malignant transformation of cells. Simply done, reliable in vitro methods with widespread availability are needed for the further evaluation and elucidation of viral delayed-type hypersensitivity (DTH) in man.

During the last decade several measures of CMI have been adapted to the study of virus infection, including the inhibition of both macrophage and explant migration by specific viral antigen (8, 10-13), blast cell transformation of sensitized lymphocytes in the presence of viral antigen (6, 7, 10, 13), and lymphocyte-target cell cytotoxicity (4, 9, 13). Although these methods have ushered in a new understanding of the host-virus relationship, they tend to be laborious and time-consuming, require expensive equipment and a high level of technical expertise, and may generate nonspecific responses produced by contaminating elements. In addition, the technical subtleties and varying sensitivities of different assay procedures have led to difficulty in reproducibility and interpretation (2).

The present study was undertaken to determine the usefulness of plaque size and number reduction as a more direct in vitro assay for viral cellular immunity. This report, we feel, describes a sensitive, specific, and uncomplicated technique for the measurement of viral DTH. The method measures the decrease in size and number of herpes simplex viral plaques in the presence of lymphocytes sensitized to that virus.

MATERIALS AND METHODS

Animals. Inbred Hartley strain female guinea pigs (400 to 600 g) (Camm Research Institute, Inc., Wayne, N. J.) were used as experimental animals in this study.

Viruses. The virus used for sensitization was herpes simplex virus (HSV) McKrae strain grown on human embryonic kidney; vaccinia virus, used to determine the specificity of plaque size reduction, was grown on L-929 cells. Soluble antigen was prepared

from HR-6 cells (HEM Research Inc., Rockville, Md.), a continuous line of human lung cells infected with HSV McKrae strain. A macroplaque herpes simplex virus (HSV-MP) was kindly supplied by Bernard Roizman (University of Chicago) and utilized on HEp-2 and Wong Kilbourne clone 22 of Chang's human conjunctiva cell monolayers. For sensitization guinea pigs were inoculated with 3×10^5 plaque-forming units of HSV in each of two footpads.

Soluble antigen. The method for preparation of soluble antigen was adapted from Lausch et al. (5). A suspension of HR-6 cells infected with McKrae HSV was disrupted with a Bronson sonifier for 30 s at a time in the cold until less than 1% intact cells remained. The suspension was centrifuged at $900 \times g$ for 20 min to pellet cell debris. The supernatant was then centrifuged at $55,200 \times g$ for 30 min at 4 C in a Beckman model L-2 ultracentrifuge to remove particulate matter. The resulting supernatant underwent ultraviolet irradiation for 90 min at a distance of 8 cm from a GE G8t5 bulb to inactivate the virus. Virus infectivity assay of the irradiated supernatant on tube cultures of human embryonic kidney revealed no cytopathological effect.

Skin testing. Soluble antigen was diluted 1:10 with physiological saline and 0.1 ml was used per skin test; protein concentration of the intradermal inoculate was 70 μg by optical density measurement at 280 nm in a Beckman DU spectrophotometer. The backs of the guinea pigs were shaved 3 h prior to skin testing. A volume of 0.1 ml of soluble antigen was injected intradermally with a 27-gauge needle into the backs of normal and sensitized guinea pigs. Uninfected HR-6 cells, at a protein concentration of 70 μg, and normal saline were used as control antigens. The skin test sites were observed for erythema and induration at 4 and 24 h after challenge. The diameter of the skin reaction was considered to be the arithmetic mean of two diameters at right angles to each other across the area of erythema. A positive dermal reaction was one with erythema greater than 10 mm after 24 h.

Preparation of cell populations. (i) Spleen cells. The method of obtaining spleen cells was adapted from Waldman and Henney (12). After scarifice of the animals by cervical fracture, a ventral incision was made through the peritoneum and the spleen was removed. After mincing the spleens, the cells were filtered through sterile gauze with the aid of Hanks balanced salt solution (HBSS) containing heparin (5 U/ml). The cell suspension was centrifuged at $250 \times g$ for 5 min at 4 C, and the sediment was resuspended and allowed to stand for 30 min at 4 C. The cells remaining in the supernatant were centrifuged at $250 \times g$ for 5 min and then resuspended in 0.83% sterile NH_4Cl for 10 min to lyse the erythrocytes. The remaining cells were centrifuged at $250 \times g$ for 5 min, washed twice with HBSS without heparin, and counted on a hemocytometer utilizing trypan blue exclusion as a test of viability. Greater than 80% of the cells were routinely viable by this criterion.

(ii) Peritoneal exudate cells. These cells were used as the source of a migrating macrophage population. Peritoneal exudates were induced in nonsensitized guinea pigs by the intraperitoneal injection of 20 ml of light sterile mineral oil 72 h before sacrifice by cervical fracture. The peritoneal cavity was washed three times with 50 ml of HBSS with heparin (5 U/ml). The washings were collected and centrifuged, and the oil and supernatant were discarded. After the cells were resuspended in HBSS without heparin and washed twice, the number of viable cells was determined by hemocytometer counting using the trypan blue exclusion test.

Inhibition of macrophage migration. The technique for macrophage migration inhibition was adapted from Waldman and Henney (12). Sensitized spleen cells were mixed with peritoneal exudate cells so that the final suspension contained 5×10^6 and 25×10^6 cells, respectively. Fifty lambda capillary tubes were filled with the cell suspension, sealed with Seal-Ease (Clay Adams, Parsippany, N.J.), centrifuged at 85 g for 4 min, cut at the cell-media interface, and placed in plastic tissue culture wells. For each experiment, six capillary tubes containing sensitized spleen cells were used. The chambers were filled with either minimal essential media with 10% fetal calf serum or the same media plus 300 μg of soluble antigen. The wells were then kept in a CO_2 incubator at 37 C for 24 h. To quantitate the extent of migration the tray of tissue culture wells containing the capillary fans was placed in an enlarger, and the magnified fans were traced on paper, cut out, and weighed. The percent of migration inhibition was calculated as: percent inhibition = (1 – mean area of migration with antigen)/(mean area of migration without antigen) $\times$ 100.

In each experiment nonsensitized cells were observed for normal migration in the presence of soluble antigen.

Plaque size and number reduction. The technique of plaque size and number reduction was adapted from Ennis (3). Conjunctiva and HEp-2 cell monolayers were grown in a 2-oz (ca. 56.7-g), 25-cm^2 plastic bottles. The tissue culture cells were grown on basal minimal media with 20% fetal calf serum and 1% glutamine, with each milliliter containing 100 U of penicillin, 100 μg of streptomycin, and 200 U of mycostatin. Both cell lines and all tissue culture media were purchased from Microbiological Associates, Bethesda, Md. The number of cells in a monolayer was determined after trypsinization by counting the trypan blue—excluding cells in one bottle. The monolayers were infected with 150 plaque-forming units of HSV-MP in 0.2 ml of HBSS and incubated at 37 C for 45 min with gentle rocking every 15 min. The monolayers were then washed twice with sterile phosphate-buffered saline. Spleen cells in a volume of 0.2 ml were then added, and the bottles were incubated for 45 min at 37 C with gentle rocking every 15 min. Control monolayers received no spleen cells, but were similarly incubated for 45 min at 37 C. At the end of the incubation period, 5 ml of minimal essential medium containing 1% methylcellulose, 10% fetal calf serum, 1% 200 mM glutamine, and sufficient 7.5% $NaHCO_3$ to bring the pH to 6.8 to 7.0 was added to each bottle. The cell layers were incubated at 37 C in a CO_2 incubator for 48 to 72 h, i.e., until plaques on control monolayers were readily visible. The methylcellulose overlay was poured off, and the cell layers were stained with 0.1% crystal violet in 95% ethanol.

The number of plaques in each monolayer was counted with the naked eye. Plaque size was assessed by placing the bottle in an enlarger and tracing on paper each of 30 or more approximately 17×-magnified plaques within a set radius in a representative area of the bottle. The diameter of each plaque was considered to be the arithmetic mean of two diameters at right angles to each other. The diameters of 30 or more plaques were averaged for each bottles; four or more bottles were used for each experiment group. Plaques were defined as localized foci of cytopathic change due to viral infection, which appeared to the naked eye as clear areas when stained. Statistical analysis was done using the analysis of variance test.

RESULTS

Initial experiments were performed to establish a sensitivity to HSV antigen in guinea pigs, as demonstrated by delayed cutaneous hypersensitivity and inhibition of macrophage migration. Ten guinea pigs sensitized with 6×10^5 plaque-forming units of HSV gave positive skin reactions to soluble HSV antigen when challenged 7 days after inoculation. The average maximal diameter of erythema was 15 mm 24 h after challenge. A nonspecific reactivity was seen with uninfected HR-6 cells which peaked at 6 h and then rapidly faded, so that at 24 h cell control reactions were uniformly less than one-fourth of the corresponding reactions to soluble antigen. Saline reactions at 24 h were negligible. In a similar manner dermal reactions in 10 guinea pigs which had been sensitized 21 days previously were found to be positive. In each group five nonsensitized control guinea pigs were found to be skin test negative (Table 1).

After induction of a hypersensitivity state that included a positive skin test to HSV antigen, a second parameter of CMI was examined. In studies of macrophage migration inhibition three groups of two animals were skin test positive to HSV soluble antigen 1 day prior to sacrifice. Each group demonstrated significant inhibition of macrophage migration in the presence of HSV antigen (Table 2). Normal migration of nonsensitized cells in each group verified the lack of toxicity of the HSV soluble antigen.

Experiments were then performed to establish the effect of sensitized and nonsensitized spleen cells on plaque size and number. Spleen cells from guinea pigs skin test positive to HSV soluble antigen were added to conjunctiva cell monolayers to which HSV-MP had been adsorbed. In a similar manner nonsensitized spleen cells were assayed for an ability to non-specifically reduce plaque size and/or number. After 48 to 72 h plaque diameters were reduced by 30% and plaque numbers by 37% on monolayers with sensitized spleen cells, as compared with plaques on monolayers without cells (P less than 0.01). Nonsensitized cells failed to significantly decrease plaque size or number in comparison with plaques on monolayers without cells (Table 3). In a like manner, plaque size and number reduction have been found with spleen cells from animals sacrificed 7, 14, and 21 days after sensitization. Plaque size and number reduction were consistently observed in the presence of immune cells on both HEp-2 and human conjunctiva cells.

To study the optimal ratio of sentized spleen cells to monolayer cells four different concentrations were used. In contrast to a ratio of one spleen cell to one conjunctiva cell, a ratio of 6:1 showed ability to reduce plaque number and size as effectively as ratios of 20:1 and 50:1

TABLE 2. *Inhibition of migration of a macrophage population by spleen cells from guinea pigs sensitized to HSV*

Group[a]	Migration inhibition in presence of antigen (%)
1	47
2	42
3	51

[a] Each group consists of the pooled spleen cells of two guinea pigs sensitized to HSV 21 days earlier.

TABLE 1. *Results of skin testing with HSV soluble antigen*

Animals tested	Avg diam of erythema 7 days after sensitization (mm)[a]		Avg diam of erythema 21 days after sensitization (mm)[a]	
	HSV soluble antigen	Uninfected HR-6 cells	HSV soluble antigen	Uninfected HR-6 cells
Guinea pigs sensitized to HSV (10)[b]	15 (±2)	3.5 (±1)	13 (±2)	2 (±1)
Nonsensitized guinea pigs (5)[b]	2.5 (±1)	3 (±1)	2.5 (±1)	2 (±1)

[a] Numbers in parentheses show range.
[b] Numbers in parentheses signify number of animals tested.

TABLE 3. *Effect of spleen cells[a] on the size and number of HSV plaques*

Cells tested[b]	Avg plaque diam (mm)	Reduction in plaque diam (%)	Avg no. of plaques/bottle	Reduction in plaque no. (%)
Spleen cells from nonsensitized guinea pigs	0.790[c]	1	145[c]	1
Spleen cells from guinea pigs 7 days after sensitization with HSV	0.581[d]	30	92[d]	37
Control	0.798		146	
Spleen cells from guinea pig 21 days after sensitization with HSV	0.642[d]	38	68[d]	41
Control	1.028		114	

[a] In a ratio of 6:1, spleen cells-monolayer cells.
[b] Control plaques were present in monolayers to which no spleen cells had been added.
[c] Not statistically significant.
[d] $P < 0.01$ by analysis of variance.

(Table 4). A ratio of 6:1 allowed full expression of the lymphocyte effect on HSV infection while using the least number of sensitized cells. In none of the groups was cytotoxicity of the monolayer cells evident at the end of 3 days.

The specificity of plaque size reduction was then demonstrated. After adsorption of vaccinia virus to a conjunctiva monolayer for 45 min, spleen cells from guinea pigs sensitized to HSV were added. After 72 h plaque size and number determination revealed that HSV-sensitized cells failed to significantly alter the spread of vaccina virus in cell culture in contrast to the controlled growth of HSV-MP (Table 5).

Finally, we endeavored to determine the cell type responsible for the in vitro inhibition of viral infection. Harvested spleen cells were allowed to stand in glass petri dishes for three successive periods of 30 min; after each period the supernatant was poured into another dish, leaving the glass-adherent cells behind. This procedure allowed us to decrease the number of macrophages to less than 5% of the total spleen cell population at the end of 90 min. The cells were then added in a ratio of six lymphocytes to one conjunctiva cell to monolayers to which HSV-MP had been adsorbed. In contrast, other bottles received sensitized spleen cells prepared in the usual manner. The macrophage-depleted spleen cell population retained its ability to reduce plaque size and number (Table 6), indicating that lymphocytes were primarily responsible for the plaque size reduction.

TABLE 4. *Ratios of spleen cells to monolayer cells in plaque size and number reduction*

Ratio of spleen cells-monolayer cells[a]	Avg plaque diam (mm)	Reduction in plaque diam (%)	Avg no. of plaques/bottle	Reduction in plaque no. (%)
1:1	0.541	6.5	158[b]	6
6:1	0.362[c]	37	113[c]	33
20:1	0.406[c]	30	109[c]	35
50:1	0.381[c]	34	112[c]	33
Control	0.578		168	

[a] Control plaques were present in monolayers to which no spleen cells had been added.
[b] Not statistically significant.
[c] $P < 0.01$ by analysis of variance.

TABLE 5. *Specificity of plaque size reduction demonstrated by utilizing HSV-sensitized spleen cells[a] against vaccinia virus*

Cells tested	Avg plaque diam (mm)	Reduction in plaque diam (%)	Avg no. of plaques/bottle	Reduction in plaque no. (%)
HSV-sensitized spleen cells versus vaccinia[b]	0.424[c]	2.5	51	2
Control	0.435		52	
HSV-sensitized spleen cells versus HSV-MP[d]	0.405[e]	30	88	38
Control	0.578		142	

[a] In a ratio of 6:1, spleen cells-monolayer cells.
[b] Vaccinia control plaques were present in monolayers to which no spleen cells had been added.
[c] Not statistically significant.
[d] HSV-MP control plaques were present in monolayers to which no spleen cells had been added.
[e] $P < 0.01$ by analysis of variance.

DISCUSSION

In vitro tests of viral CMI currently play an important role in delineating the interplay between host and virus in the disease state. They are methodological variations measuring either lymphocyte-mediated cytotoxicity, lymphokine production, especially migration inhibition factor, or lymphocyte transformation. The relationship between these tests and actual cellular immunity to viruses is ill-defined, resulting at

TABLE 6. *Plaque size and number reduction with lymphocytes*

Spleen cells	Avg plaque diam (mm)	Reduction in plaque diam (%)	Avg no. of plaques/ monolayer	Reduction in plaque no. (%)
Spleen cell sensitized to HSV 95% lymphocytes[a]	0.410[b]	45	148	32
Spleen cells sensitized to HSV[c]	0.508	32	157	28
Controls[d]	0.747		218	

[a] Partially purified suspension of lymphocytes used in a ratio of 6:1.

[b] $P < 0.01$ by analysis of variance.

[c] Used in a ratio of 6:1.

[d] Control plaques were present in monolayers to which no spleen cells had been added.

times in conflicting data (2). Due to the difficulties of interpretation, as well as the long hours, special equipment, and technical expertise needed to carry out these tests, their availability and usefulness are limited. Measures of viral CMI are needed that are sensitive, technically simple, and, perhaps most important, directly related to cellular antiviral activity. To determine if plaque size and number reduction might serve this function we adapted a system described by Ennis (3) and endeavored to develop an uncomplicated methodology in guinea pigs which would correlate well with other in vitro tests of CMI.

Infectious virus particles in a cell monolayer have the ability to produce cytopathic changes in localized foci called plaques. The size of plaques under an agar or methylcellulose overlay is dependent on the spread of virus between contiguous monolayer cells. The spread of viral infection is inhibited if lymphocytes sensitized to the particular virus are present in sufficient numbers. In our studies lymphocytes sensitized to HSV, in contrast to nonsensitized cells, were shown to decrease the number and area of plaques resulting from HSV-MP on both HEp-2 and conjunctiva cell monolayers, thereby demonstrating the potential usefulness of this test in man.

Sensitized spleen cells appear to show an immune-specific inhibition of spread of HSV infection in cell monolayers. Spleen cells from HSV-sensitized guinea pigs decrease the size and number of the foci of infection due to HSV-MP, but not to vaccinia. These findings are complemented by Ennis' work (3), in which spleen cells from animals immunized with influenza virus failed to significantly reduce herpes simples virus plaque size. By utilizing the specificity of plaque size reduction a better understanding of the actual course of events in the host-virus interaction may be achieved.

In lieu of any single test of viral CMI that fully reflects the host-immune status, several parameters of CMI must be correlated with one another. Plaque size and number reduction compared well with skin testing and macrophage migration inhibition in the guinea pig. The capacity of spleen cells from sensitized animals to limit the size and number of viral plaques by day 7 corresponds well to the development of delayed cutaneous hypersensitivity. All three methods were positive 21 days after antigen stimulation. Each test was found to be negative in nonsensitized animals.

The in vitro interaction between sensitized lymphocytes and an actively growing virus in many respects resembles the in vivo state. The mechanisms by which sensitized cells restrict the growth of a viral infection in vitro have been studied by Ennis (3). Using mouse spleen cells against HSV on L-929 and human conjunctiva under an agar overlay, Ennis found a decrease in plaque diameter but not in plaque number. However, by using a microscope to count plaques he could enumerate the very small foci of viral infection missed by the eye alone and possibly overlooked in our study. In this study, as in that of Ennis, a macrophage-depleted spleen cell population retained the ability to limit the spread of HSV infection. Studies are currently underway in our laboratory to further investigate the ability of sensitized lymphocytes to limit the spread of viral infection.

The technique of plaque size and number reduction provides a potent tool for measuring viral CMI in vitro. The data presented show that the cellular immune reaction to HSV infection in the guinea pig can be measured in vitro by this method. Plaque size and number reduction correlate well with skin testing and inhibition of macrophage migration, in addition to being specific, sensitive, and reproducible. Moreover, this in vitro method is a simpler and more straightforward measurement of antiviral CMI—in many respects resembling the in vivo state.

ACKNOWLEDGMENTS

The technical assistance provided by Suzanne Zam, Pauline Jones, and Maxie Hicks is gratefully acknowledged.

This work was supported by Public Health Service grants no. EY-00007, EY-00446, and EY-00033 from the National Eye Institute.

LITERATURE CITED

1. Allison, A. C. 1972. Immunity and immunopathology in virus infections. Ann. Inst. Pasteur (Paris) **123**:585-608.

2. Bloom, B. R, R. Ceppellini, J. C. Cerottini, J. R. David, H. Kunkel, M. Landy, H. S. Lawrence, R. Maini, V. Nussenzweig, P. Perlman, F. Rosen, L. Spitler, and J. Zabriskie. 1973. *In vitro* methods in cell-mediated immunity: a progress report. Cell. Immunol. **6**:331-347. (Special Feature)
3. Ennis, F. A. 1973. Host defense mechanisms against herpes simplex virus. Infect. Immunity **7**:898-904.
4. Kolb, W. P., T. W. Williams, and G. A. Granger. 1971. Lymphocyte activation and lymphotoxin production, p. 333-342. *In* B. R. Bloom and P. Glade (ed.), *In vitro* methods in cell-mediated immunity. Academic Press Inc., New York.
5. Lausch, R. N., J. S. Swyers, and H. E. Kaufman. 1966. Delayed hypersensitivity to herpes simplex virus in the guinea pig. J. Immunol. **96**:981-987.
6. Olson, G. B., P. B. Dent, W. E. Rawls, M. A. South, J. R. Montgomery, J. L. Melnick, and R. A. Good. 1968. Abnormalities of *in vitro* lymphocyte responses during rubella virus infections. J. Exp. Med. **128**:47-68.
7. Oppenheim, J. J. 1968. Relationship of *in vitro* lymphocyte transformation to delayed hypersensitivity in guinea pigs and man. Fed. Proc. **27**:21-28.
8. Pekarek, J., J. Svejcar, V. Onka, and H. Zavadova. 1967-68. The use of an *in vitro* test of delayed type hypersensitivity for the detection of specific tumor antigen of transplantation type induced by sv 40 virus. Z. Immunitaetsforsch. Allerg. Klin. Immunol. **134**:449-461.
9. Speel, L. F., J. E. Osborn, and D. L. Walker. 1968. An immuno-cytopathogenic interaction between sensitized leukocytes and epithelial cells carrying a persistent noncytocidal myxovirus infection. J. Immunol. **101**:409-417.
10. Thor, D. E., R. E. Jureziz, S. R. Veach, E. Miller, and S. Dray. 1968. Cell migration inhibition factor released by antigen from human peripheral lymphocytes. Nature (London) **219**:755-757.
11. Tompkins, W. A. F., C. Adams, and W. E. Rawls. 1970. An *in vitro* measure of cellular immunity to fibroma virus. J. Immunol. **104**:502-510.
12. Waldman, R. H., and C. S. Henney. 1971. Cell-mediated immunity and antibody responses in the respiratory tract after local and systemic immunization. J. Exp. Med. **134**:482-494.
13. Wilton, J. M. A., L. Ivanyi, and T. Lehner. 1972. Cell-mediated immunity in herpesvirus hominis infections. Brit. Med. J. **1**:723-726.