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# Lysis of Cytomegalovirus-Infected Human Fibroblasts and Transformed Human Cells by Peripheral Blood Lymphoid Cells from Normal Human Donors (39650)[1]

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The cellular immune response to cytomegalovirus (CMV) infection in man has not been well defined. However, the importance of cellular defenses is suggested by the association of severe CMV infections with immune-deficiency states and immunosuppression (1).

Several studies have delineated various cellular cytotoxic mechanisms capable of the destruction of cells infected by other members of the *Herpes* virus group. For example, antibody-dependent cell-mediated cytotoxicity (ADC) has been implicated in the immune elimination of *Herpes simplex* virus (HSV)-infected cells (2, 3). Hutt *et al.* (4) demonstrated the cytotoxicity of normal human leukocytes for Epstein-Barr virus (EBV)-transformed human lymphoblastoid cell lines which was increased in patients convalescing from infectious mononucleosis. Our experiments were undertaken to evaluate the possible role of various cell-mediated cytotoxic mechanisms in the destruction of target cells infected with CMV.

In this study, using human peripheral blood mononuclear cells from normal nonimmune donors, we observed the unexpected lysis of CMV-infected human fibroblasts. The observed lysis of CMV-infected human fibroblasts appeared to resemble descriptions of "spontaneous" cytolysis of several cell lines by nonimmune lymphocytes (5–9). Therefore, lysis of CMV-infected fibroblasts is compared with activity against three "transformed" human cell lines.

*Materials and methods.* Human neonatal foreskin fibroblast (FS) cell cultures and stocks of CMV strain AD-169 were prepared as previously described (10). During the course of the experiments, four different preparations of FS cell cultures were used. CMV-infected FS cells were prepared in two manners. Monolayers of FS were infected with trypsin-dispersed infected cells (at a 1:3 ratio of infected: noninfected cells) and used 3 to 6 days later when infection was nearly complete. For infection with free virus, 24-hr-old FS cells were infected at a multiplicity of infection of 1 and utilized 24 to 72 hr later. Uninfected FS cells were grown in a similar manner. These, as well as the HEp-2 and Chang cell lines, were grown in Eagle's Minimal Essential Medium supplemented with 1% glutamine, 100 U/ml penicillin, 100 μg/ml of streptomycin, 2.5 μg/ml of amphotericin B, and 10% fetal calf serum. The omission of amphotericin B in some experiments did not alter the observed cytolysis. Suspension cultures of the P2HR-1 cells (11), an EBV-transformed lymphoid cell line, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum.

Monolayer cultures of target cells were suspended by incubation with 0.05% trypsin in 0.05% EDTA for 5 min. All futher cell incubations were performed in RPMI 1640 medium (Hepes-buffered) supplemented with 10 m*M* L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 5% agamma or normal fetal calf serum. Viable target cells ($5 \times 10^6$/ml) in 1 ml of medium were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 hr at 37°. After washing four times, target cells were counted using trypan blue, and $5 \times 10^4$ target cells were dispensed into tubes in a 0.1-ml volume. The incubation tubes were flat-bottomed 10 ×

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47-mm glass (Becton Dickinson, Rutherford, New Jersey), which had been washed with deionized water and autoclaved before use. Tubes with labeled target cells were incubated at 37° for 3 hr to permit formation of a stable monolayer.

Human peripheral blood was obtained from 14 normal donors with complement fixation (CF) titers of less than 1:8 to CMV and one patient convalescing from CMV mononucleosis (documented by a fourfold CF titer rise). To verify further the immunologic status of donors, sera were tested by the more sensitive (12, 13) indirect fluorescent antibody (IFA) technique (10). This was modified from our earlier procedures by using 24-hr-old CMV-infected fibroblasts to minimize confusion of anti-viral nuclear antibodies with cytoplasmic IgG-Fc receptors, which become maximal at 72 hr after infection. Of 11 donors tested by IFA, only one was positive, at a 1:4 dilution. Fresh cord blood was obtained after normal deliveries. Most blood was defibrinated with glass beads, but some samples were heparinized with no discernible effect on results. Mononuclear cells were separated using Hypaque-Ficoll as outlined by Böyum (14). In some cases, phagocytic cells were eliminated by modifications of the techniques of Lundgren *et al.* (15). Blood was mixed in a siliconized flask with 1 mg/ml of carbonyl iron (GAF, Manchester, England) which had been sonicated just before use. Flasks were incubated on a rocking platform for 60 min at 37° and were swirled by hand every 5 min to ensure further, mixing. After repeated passages through a magnetic field, the remaining lymphocytes were separated by Hypaque-Ficoll. After neutral red or latex particle ingestion, only 0.5–1.5% phagocytic cells remained. T-cell enriched populations were prepared according to the technique of Julius *et al.* (16). After Hypaque-Ficoll separation, mononuclear cells were diluted in MEM containing 5% fetal calf serum and incubated at 37° for 45 min in columns containing washed, sterile, nylon wool (LP-1 Leuko-Pak Leukocyte Filters, Fenwall Laboratories, Morton Grove, Ill.). Effluent cells were washed three times before use.

Leukocytes were suspended in RPMI 1640 (Hepes-buffered and supplemented as outlined above) with 5% fetal calf serum and added to $^{51}$Cr-labeled target cells. The final volume in each tube was adjusted to 0.8 ml with medium. After an 18-hr incubation at 37°, 0.4 ml of the supernate was carefully removed from each tube, and counted in a gamma counter. The total releasable radioactivity from each group of target cells was determined using specimens lysed with 1% Triton X-100. Spontaneous $^{51}$Cr release was determined from tubes containing no effector cells. The percentage of specific lysis was computed as: (mean $^{51}$Cr release in test group − mean spontaneous $^{51}$Cr release)/(mean $^{51}$Cr release after total lysis − mean spontaneous $^{51}$Cr release) × 100. All experiments were performed in triplicate.

*Results.* In initial experiments using leukocytes from 14 normal donors. the maximum specific lysis of 32.3 ± 4.2% (mean ± SE) CMV-infected FS cells was observed. Maximum cytolysis occurred at effector:target cell ratios of 100–400:1. However, at the target cell numbers ($5 \times 10^3$ cells/tube) used in these experiments, it was found that uninfected FS cells spontaneously released unacceptably high percentages of $^{51}$Cr. Using a 10-fold greater density of target cells ($5 \times 10^4$/tube), both CMV-infected and uninfected FS cells proved more stable; spontaneous $^{51}$Cr release, in the absence of effector leukocytes, was 15.9–32.7% after 18 hr.

Using this greater target cell density, CMV-infected FS cells were lysed by leukocytes from normal adults, whereas uninfected fibroblasts were not (Table I). Cytolysis of CMV-infected FS cells was proportional to the number of effector cells present at effector:target cell ratios of 6.25–50:1, but declined at higher ratios. An examination of the time course for the development of susceptibility to cytotoxicity after CMV infection demonstrated lysis as early as 24 hr postinfection. Similar levels of lysis were observed in 24- and 72-hr-old infected FS cells (50.1 and 58.2%, respectively).

In order to determine if the observed cytotoxicity is present from birth, lysis of CMV-infected FS cells by cord blood mononuclear leukocytes was studied. It was found

TABLE I. CYTOLYSIS OF CMV-INFECTED AND UNINFECTED FIBROBLASTS BY LEUKOCYTES.

| Target cell | Source of leukocytes (number tested) | Specific lysis at the following ratios of leukocytes:target cells[a] | | | |
|---|---|---|---|---|---|
| | | 50:1 | 25:1 | 12.5:1 | 6.25:1 |
| Fibroblasts | Normal subjects (14) | 2.6 ± 0.8 | 4.8 ± 0.9 | 4.6 ± 1.4 | 5.9 ± 2.3 |
| CMV-infected fibroblasts | Normal subjects (14) | 53.8 ± 3.9 | 42.3 ± 4.4 | 21.6 ± 3.8 | 11.8 ± 2.1 |
| CMV-infected fibroblasts | Cord blood (3) | 6.7 ± 1.7 | 8.9 ± 3.2 | 6.9 ± 4.8 | 4.3 ± 1.5 |

[a] Percentage of $^{51}Cr$ release ± SE.

that cord blood leukocytes induced only minimal lysis of CMV-infected fibroblasts (Table 1). Since active CMV infection might be expected to enhance cellular immunity to CMV-infected target cells, we studied one patient convalescing from CMV mononucleosis. At the time of study (4 weeks after onset), his leukocytes lysed CMV-infected cells at the mean rate of leukocytes for normal subjects.

Because of the known importance of ADC in destruction of HSV-infected cells, we studied the role of specific antibody in this reaction. Anti-CMV antibody (1:10 dilutions of two sera from convalescent patients with 1:320 and 1:1024 CF titers) did not augment cytotoxicity. In contrast, Chang cell lysis was markedly augmented by rabbit anti-Chang cell antibodies (two- to threefold increased lysis at effector:target cell ratios of 25–50:1, as well as significant antibody-dependent lysis at ratios as low as 3–7:1). Moreover, we noted no reduction in the levels of cytotoxicity of CMV-infected cells when the reactions were carried out in the presence of specific viral antibody. Because CMV infection induces the development of an IgG-Fc receptor in FS cells (10), we studied the possibility that γ-globulin present in fetal calf serum might mediate ADC. In eight experiments using agamma fetal calf serum substituted for fetal calf serum, no difference in cytotoxicity was noted.

Leukocytes were further separated in order to study the nature of the effector cell active in the spontaneous lysis of CMV-infected target cells. In two experiments, treatment of Hypaque–Ficoll-separated leukocytes with carbonyl iron and a magnet to remove phagocytic cells did not change cytotoxicity. Hypaque–Ficoll-separated leukocytes were then passed through a nylon wool column. This almost totally depleted immunoglobulin-bearing cells (determined by direct immunofluorescence using a polyvalent goat anti-human immunoglobulin serum). In two experiments, this resulted in 52 and 60% decreases in cytotoxicity compared with Hypaque–Ficoll-separated leukocytes.

The lymphocyte-mediated spontaneous lysis of the CMV-infected FS cells observed in these studies appeared to resemble descriptions of lysis of transformed cell lines. Therefore, further studies were done to compare the lysis of different cell lines by leukocytes from normal donors. In general, the lysis of CMV-infected FS cells was in the same range as the lysis of three transformed human cell lines, i.e., HEp-2, Chang, and P3HR-1 (Table II).

*Discussion.* The results presented here demonstrate that human peripheral blood leukocytes from nonimmune donors are capable of injuring CMV-infected human fibroblasts. No such cytotoxicity was observed when similar effector cells were combined with uninfected target cells. Effector cells from all 14 normal donors tested demonstrated this nonspecific cytotoxicity. Moreover, the cytotoxic effect did not appear to be mediated by T cells, and it occurred independent of the presence of specific anti-viral antibodies. These findings suggest that this activity is not directed against the viral antigens displayed on the surface of CM-infected cells. Rather, it may be directed against some modification of the cell surface induced as a result of the virus infection.

Recent findings from this laboratory (10) and others (17) have shown that CMV infection induces the formation of an IgG-Fc receptor in human cells. This IgG-Fc recep-

TABLE II. COMPARISON OF CYTOLYSIS OF CMV-INFECTED FIBROBLASTS WITH CYTOLYSIS OF HUMAN CELL LINES BY LEUKOCYTES FROM NORMAL SUBJECTS.

| Target cell (number of experiments) | Specific lysis at the following ratios of leukocytes:target cells[a] | | | |
|---|---|---|---|---|
| | 50:1 | 25:1 | 12.5:1 | 6.25:1 |
| CMV-infected fibroblasts (14) | 53.8 ± 3.9 | 42.3 ± 4.4 | 21.6 ± 3.8 | 11.8 ± 2.1 |
| HEp-2 (14) | 41.2 ± 4.5 | 26.3 ± 5.4 | 16.9 ± 5.4 | 2.1 ± 5.9 |
| Chang (2) | 42.5 | 29.2 | 11.5 | 2.9 |
| P3HRI, EBV-transformed (1) | 45.2 | 41.3 | 29.9 | 15.9 |

[a] Percentage of $^{51}Cr$ release ± SE.

tor is present on cell surfaces and has been detected in tissue culture fluids as early as 24 hr postinfection (18). If IgG aggregates or immune complexes are present and react with IgG-Fc receptors on CMV-infected cells, they might then be expected to react with the IgG-Fc receptors of ADC cells. Therefore, one possible mechanism for this apparent natural cytotoxicity may be through the linking of ADC effector cells to virus-infected target cells. In fact, such a mechanism has recently been proposed to account for the natural cytotoxic effect demonstrated by normal murine macrophages against HSV-infected mouse neuroblastoma cells (19). However, the mediation of ADC by IgG complexes in our system would seem unlikely, based on our findings of equal levels of cytotoxicity when experiments were conducted in the presence or absence of IgG, i.e., agamma calf serum. Nevertheless, this possibility has not been totally excluded since the effector cell population contained B cells which conceivably, might have produced IgG. The hypothetical role of IgG microaggregates and ADC effector cells in this system would be consistent with our finding that nylon wool column-purified lymphocyte populations depleted of B lymphocytes demonstrated a 50 to 60% decrease in cytotoxic activity. We are currently studying ADC as a possible mechanism for natural cytotoxicity in this system.

The cytotoxicity developed by human leukocytes against CMV-infected cells appears to be similar to the nonspecific cytotoxicity observed with the three transformed human cell lines examined. On the basis of the present data, we are unable to conclude that the surface signal produced by the CMV infection is similar to that present on human transformed cell lines. However, it is of interest to note that CMV-infected cells demonstrate some alterations of the cell surface which are characteristic of transformed cells. Thus, although uninfected human fibroblasts are unable to replicate in media containing agarose, CMV-infected human diploid fibroblasts acquire the ability to grow into small colonies in the presence of agarose (20). Furthermore, human fibroblasts infected with CMV assume an epithelioid conformation and continue active division during the first few days following infection, prior to undergoing cellular lysis (personal observations). CMV infection stimulates host cell DNA synthesis, an important characteristic shared with oncogenic DNA viruses (21). Indeed, CMV has been shown capable of inducing oncogenic transformation of hamster embryo fibroblasts (22) and human embryo lung cells (23). On this basis, it is tempting to speculate that the natural cytolysis of CMV-infected cells is directed against an early alteration of cell membranes common to several types of human transformed cells.

Two serologic parameters failed to detect evidence of previous immunologic experience with CMV in all but one normal donor. Nevertheless, lymphocytes from that donor and one CMV convalescent donor did not demonstrate greater cytotoxicity against CMV-infected fibroblasts than cells from nonimmune donors. Cytolysis of CMV-infected fibroblasts closely paralleled the lysis of three transformed cell lines. On this basis, the ineffectiveness of peripheral blood leukocytes obtained from cord blood to serve as cytotoxic effector cells suggests the involvement of an immunologic response to altered cell surface antigens rather than a direct response to viral antigens. Alternatively, this may be explained by the

functional prematurity of the effector cell population responsible for this natural cytotoxicity. However, it should be noted that these results differ from results reported by Pross and Jondal (6) who noted the cytolysis of human cell lines by cord blood lymphoid cells, though their data is not reported in detail.

The biological significance of spontaneous cytotoxicity is unclear. Nevertheless, the failure of leukocytes to lyse normal fibroblasts in our studies suggests a possible role for spontaneous cytotoxicity in normal surveillance against virus-infected and transformed cells in man.

*Summary.* Peripheral blood leukocytes from 14 healthy, nonimmune, human donors were capable of destroying CMV-infected human fibroblasts, but non uninfected fibroblasts. Antibody did not appear to mediate the response. Cytotoxicity in a similar range was noted when three transformed human cell lines were used as target cells. Unlike adult leukocytes, leukocytes separated from three samples of cord blood were ineffective in destroying CMV-infected target cells.

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1. Weller, T. H., N. Engl. J. Med. **285,** 203, 267 (1971).
2. Rager-Zisman, B., and Bloom, B. R., Nature (London) **251,** 542 (1974).
3. Shore, S. L., Black, C. M., Melewicy, F. M., Wood, P. A., and Nahmias, A. J., J. Immunol. **116,** 194 (1976).
4. Hutt, L. M., Huang, Y. T., Dascomb, H. E., and Pagano, J. S., J. Immunol. **115,** 243 (1975).
5. Jondal, M., and Pross, H., Int. J. Cancer **15,** 596 (1975).
6. Pross, H., and Jondal, M., Clin. Exp. Immunol. **21,** 226 (1975).
7. Takasugi, M., Mikey, M. R., and Terasaki, P. I., Cancer Res. **33,** 2898 (1973).
8. Peter, H. H., Pavie-Fischer, J., Fridman, W. H., Aubert, C., Cesarini, J-P., Roubin, R., and Kourilsby, F. M., J. Immunol. **115,** 539 (1975).
9. Rosenberg, E. B., McCoy, J. L., Green, S. S., Donelly, F. C., Siwarsky, D. F., Levine, P. H., and Herberman, R. D., J. Nat. Cancer Inst. **52,** 345 (1974).
10. Keller, R., Peitchel, R., Goldman, J., and Goldman, M., J. Immunol. **116,** 772 (1976).
11. Hinuma, Y., and Grace, J. T., Proc. Soc. Exp. Biol. Med. **124,** 107 (1967).
12. Betts, R. F., George, S. D., Rundell, B. B., Freeman, R. B., and Douglas, R. G., Jr., J. Clin. Microbiol. **4,** 151 (1976).
13. Stagno, S., Reynolds, D., Tsiantos, A., Fuccillo, D. A., Smith, R., Tiller, M., and Alford, C. A., Jr., J. Inf. Dis. **131,** 522 (1975).
14. Böyum, A., Scand. J. Clin. Lab. Invest. Suppl. **97,** 77 (1968).
15. Lundgren, G., Zukoski, C. F., and Möller, G., Clin. Exp. Immunol. **3,** 817 (1968).
16. Julius, M. H., Simpson, E., and Herzenberg, L. A., Eur. J. Immunol. **3,** 645 (1973).
17. Furukawa, T., Hornberger, E., Sakuma, S., and Plotkin, S. A., J. Clin. Microbiol. **2,** 332 (1975).
18. Sakuma, S., Furukawa, T., and Plotkin, S. A., Abstracts of the Annual Meeting of the American Society for Microbiology, p. 248 (1976).
19. Rager-Zisman, B., Grose, C., and Bloom, B. R., Nature (London) **260,** 369 (1976).
20. Lang, D. J., Montagnier, L., and Laterjet, R., J. Virol. **14,** 327 (1974).
21. St. Jeor, S. C., Albrecht, T. B., Funk, F. D., and Rapp, F., J. Virol. **13,** 353 (1974).
22. Albrecht, T., and Rapp, F., Virology **55,** 53 (1973).
23. Geder, L., Lausch, R., O'Neill, F., and Rapp, F., Science **192,** 1134 (1976).

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