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NOTES

Use of Cryopreserved Virus-Infected Target Cells in a Lymphocytotoxicity ^{51}Cr Release Microassay for Cell-Mediated Immunity to Cytomegalovirus

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A new ^{51}Cr-release microassay is described for the measurement of cell-mediated immunity to cytomegalovirus, using cryopreserved target cells acutely infected with cytomegalovirus.

The study of cell-mediated immunity (CMI) to viruses is assuming increasing clinical importance because of its role in resistance to many viral infections (1). The availability of a lymphocytotoxicity ^{51}Cr-release assay using target cells persistently infected with viruses has provided a convenient tool for the evaluation of CMI to viruses in man (2-4). Because of the nonavailability of cell lines persistently infected with cytomegalovirus (CMV), we have searched for alternate procedures. We report here, as an alternative, the use of cryopreserved cells acutely infected with CMV as target cells in the ^{51}Cr-release assay.

The method for the preparation and monitoring of the infected target cells for use in the assay is schematically represented in Fig. 1. The seed virus used in these studies was the AD-169 strain of CMV (5). Monolayer cultures of WI-38 (diploid human embryonic lung cells) each containing approximately 6×10^6 cells were inoculated with CMV at a multiplicity of infection of 1:1. After 24 h of incubation at 37 C, the monolayer cultures were washed with ethylenediaminetetraacetic acid (1:5,000) and dissociated with 0.125% pancreatin. After centrifugation at $150 \times g$ for 10 min, the packed cells were resuspended to a final concentration of 2×10^6 cells/ml in cryoprotective media (Eagle minimal essential medium [MEM] prepared with Hanks balanced salt solution supplemented with 20% heat-inactivated fetal bovine serum) containing 7.5% dimethyl sulfide. The cells were frozen in 4-ml aliquots in screw-cap Pyrex tubes (16 by 75 mm) in a Linde BF-4 controlled-rate freezing unit at a rate of 1 C per min to −30 C. At this point, the rate was increased to 10 C per min; at −150 C the tubes were removed from the freezing chamber and transferred for storage to the vapor phase of a liquid nitrogen refrigerator. Control uninfected WI-38 cells were similarly processed.

For the evaluation of infectivity, a sample of the frozen pool was inoculated into human foreskin cell cultures (MA-184) (Fig. 1). After inoculation, the cultures were maintained with weekly feedings of a maintenance medium consisting of MEM with 2% heat-inactivated fetal bovine serum. The cultures were observed over a 6-week period for typical CMV cytopathogenicity, and viral infectivity was calculated as 4.37×10^6 mean tissue culture infective doses per ml. The degree of cell surface antigens was also determined on samples of the infected target cells by indirect immunofluorescence (Fig. 1). This was determined on cells which were grown in culture over a 24- to 96-h period. After fixation with 95% methanol, over 85% of the adherent target cell population revealed fluorescence. The control cells were negative in both tests of infectivity and immunofluorescence.

On the day of the lymphocytotoxicity assay, the required number of vials of virus-infected and control cells were rapidly thawed at 37 C and diluted with 10 volumes of MEM supplemented with 10% fetal bovine serum. The cells were washed and centrifuged at $150 \times g$ three

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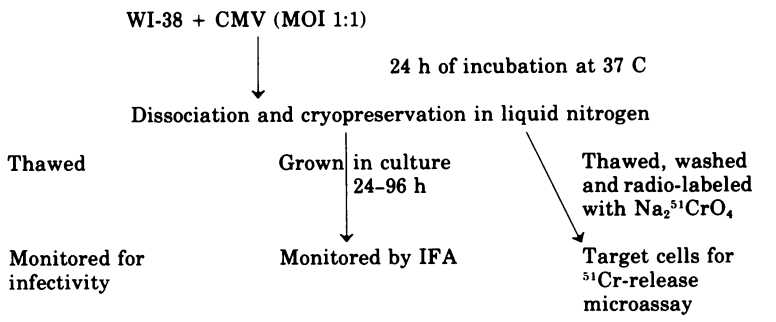

FIG. 1. *Flow sheet illustrating the preparation and monitoring of CMV-infected target cells for use in the ^{51}Cr-release lymphocytotoxicity microassay. MOI, Multiplicity of infection; IFA, indirect immunofluorescence.*

times and resuspended to a final concentration of 10^6 cells/ml for ^{51}Cr labeling by methods described previously (3). Briefly, control and infected cells were radiolabeled by incubating 10^6 cells with 100 μCi of $Na_2{}^{51}CrO_4$ in a total volume of 1 ml for 30 min at 37 C in a 5% CO_2 atmosphere, maintaining constant agitation on a rocker platform. The reaction was stopped by adding 50 ml of MEM at 4 C, and the cells were washed twice to eliminate unbound chromium. The cells were resuspended in 5 ml of cold medium and kept at 4 C for 90 min. The cells were then washed twice in 50 ml of cold medium and adjusted to a final concentration of 5×10^4 cells/ml for use in the lymphocytotoxicity assay (3).

Purified suspensions of mononuclear cells were prepared by the hypaque-ficoll method and the ^{51}Cr-release assay was performed as previously described (3). Test cultures consisted of 5×10^5 lymphocytes and 5×10^3 target cells in 0.2 ml of medium, giving an attacker-to-target cell ratio of 100:1. Additional control cultures consisted of target cells without lymphocytes. Experiments were performed in triplicate. The percentage of release for each cell line was calculated as follows:

$$\text{Release (\%)} = \frac{\text{(Counts per minute of } ^{51}\text{Cr released from target cells and lymphocytes during incubation)} - \text{(Counts per minute of } ^{51}\text{Cr released spontaneously from target cells alone during incubation)}}{\text{(Total } ^{51}\text{Cr releasable)} - (^{51}\text{Cr released at 0 time of incubation)}} \times 100$$

The specific immune release (SIR) was obtained by subtracting the percentage of release for control cells from that for CMV-infected cells. Since optimal incubation periods varied with the individual tested, determinations were carried out at more than one incubation time. We have previously described that most reactions are maximal at 18 or 24 h, and consequently these two time periods were chosen. The higher value of the 18- or 24-h release for either of these time periods was used in the calculation of SIR.

Based upon the results obtained from several test systems thus far evaluated, SIR values below 5% have been found to be significant in this assay.

Serum antibody was determined by the indirect hemagglutination test for CMV as described previously (6). For these studies four seronegative and 10 seropositive individuals were studied.

The results of these studies are shown in Table 1. Of 14 healthy subjects, 4 without detectable indirect hemagglutination serum CMV antibody titers did not show significant SIR. In contrast, all 10 subjects with positive indirect hemagglutination titers to CMV had

TABLE 1. *Comparison of humoral and cell-mediated immunity to CMV in healthy subjects*

Subject	Age	CMV indirect hemagglutination titer	Specific CMV immune release (%)	
			18 h	24 h
L.M.	24	<1:8	0	0
W.S.	23	<1:8	0	0
C.M.	8	<1:8	3.4	0
L.F.	29	<1:8	0	3.1
R.B.	27	1:8	24.6	0
B.H.	25	1:8-16	0	10.3
B.B.	26	1:16-32	1.4	37.7
A.H.	30	1:32	23.0	0
D.H.	32	1:32	7.0	12.1
D.K.	30	1:32-64	19.2	0
E.O.	28	1:32-64	4.9	0
Y.H.	29	1:64	16.6	0
J.B.	40	1:64-128	0	6.4
J.W.	31	1:64-128	3.8	20.8

significant SIR ranging from 4.9% to 37.7%.

The present studies describe a new test of CMI to CMV using a ^{51}Cr-release assay. These studies also demonstrate that target cells infected with CMV in a potentially cytolytic infection can be arrested at an early stage of acute viral cell interaction (24 h) by means of cryopreservation, and that these cells can be used as targets for the microassay of CMI to viruses. The availability of this technique obviates the necessity for the use of chronically infected cell lines. The method may also prove to be useful for the study of CMI to other viruses for which chronically infected cell lines are not yet established.

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