are at work for cellular antigens also. This confirms their importance in possible manipulations of anti-tumor immune response [36, 37].

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5. References

1 Schierman, L.W. and McBride, R.A., *Science* 1967. *156*: 658.

2 Stimpfling, J.H. and McBroom, C.R., *Transplantation* 1971. *11*: 87.

3 Watkins, J.F. and Chen, L., *Nature* 1969. *223*: 1018.

4 Britton, S., *Transplant. Rev.* 1971. *7*: 146.

5 Colnaghi, M.I. and Della Porta, G., *J. Nat. Cancer Inst.* 1973. *50*: 173.

6 Di Marco, A.T., Franceschi, C. and Prodi, G., *Eur. J. Immunol.* 1972. *2*: 240.

7 Boyse, E.A., Bressler, E., Iridani, C.A. and Lardis, M., *Transplantation.* 1970. *9*: 339.

8 Greaves, M.F. and Raff, M.C., *Nature-New Biol.* 1971. *233*: 239.

9 Iverson, G.M. and Lindenmann, J., *Eur. J. Immunol.* 1972. *2*: 195.

10 Czajkowski, N.P., Rosenblatt, M., Wolf, P.L. and Vazquez, J., *Lancet* 1967. *ii*: 905.

11 Braun, W., Plescia, O.J., Raskova, J. and Webb, D., *Isr. J. Med.* 1971. *7*: 72.

12 Kurth, R. and Bauer, H., *Eur. J. Immunol.* 1973. *3*: 95.

13 Lindenmann, J. and Klein, P.A., *J. Exp. Med.* 1967. *126*: 93.

14 Kobayashi, H., Sendo, F., Kaji, H., Shirai, T., Saito, H., Takeichi, N., Hosokawa, M. and Kodama, T., *J. Nat. Cancer Inst.* 1970. *44*: 11.

15 Boone, C., Blackman, K. and Brandchaft, P., *Nature* 1971. *231*: 265.

16 Beverley, P.C.L., Lowenthal, R.M. and Tyrrell, D.A.J., *Int. J. Cancer* 1973. *11*: 212.

17 Martin, W.J., Wunderlich, J.R., Fletcher, F. and Inman, J.K., *Proc. Nat. Acad. Sci. US* 1971. *68*: 469.

18 Mitchison, N.A., *Transplant. Proc.* 1970. *2*: 92.

19 McBride, R.A. and Schierman, L.W., *J. Immunol.* 1973. *110*: 1710.

20 Boone, C.W., Paranjpe, M., Orme, T. and Gillette, R., *Int. J. Cancer* 1974. *13*: 543.

21 McCullagh, P.J., *J. Exp. Med.* 1970. *132*: 916.

22 Katz, D.H., Paul, W.E., Goidl, E.A. and Benacerraf, B. *J. Exp. Med.* 1971. *133*: 169.

23 Kreth, H.W. and Williamson, A.R., *Nature* 1971. *234*: 454.

24 Mitchison, N.A., *Eur. J. Immunol.* 1971. *1*: 18.

25 Bretscher, P.A. and Cohn, M., *Nature* 1968. *220*: 444.

26 Fujimoto, S., Chen, C.H., Sabbadini, E. and Sehon, A.H., *J. Immunol.* 1973. *111*: 1093.

27 Uhr, J.W. and Möller, G., *Advan. Immunol.* 1968. *8*: 81.

28 Wigzell, H., *J. Exp. Med.* 1966. *124*: 953.

29 Rowley, D.A. and Fitch, F.W., *J. Exp. Med.* 1964. *120*: 987.

30 Möller, G., Andersson, J. and Sjöberg, O., *Cell. Immunol.* 1972. *4*: 416.

31 Schmidtke, J.R. and Dixon, F.J., *J. Exp. Med.* 1972. *136*: 392.

32 Louis, J.A., Chiller, J.M. and Weigle, W.O., *J. Expr. Med.* 1973. *138*: 1481.

33 Schrader, J.W., *Eur. J. Immunol.* 1974. *4*: 20.

34 Katz, D.H., Paul, W.E. and Benacerraf, B., *J. Immunol.* 1971. *107*: 1319.

35 Devlin, R.G., McCurdy, J.D. and Baronowsky, P.E., *J. Exp. Med.* 1974. *139*: 230.

36 Katz, D.H., Ellman, L., Paul, W.E., Green, I. and Benacerraf, B., *Cancer Res.* 1972. *32*: 133.

37 Ellman, L., Katz, D.H., Green, I., Paul, W.E. and Benacerraf, B., *Cancer Res.* 1972. *32*: 141.

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Target cell-dependent T cell-mediated lysis of vaccinia virus-infected cells

Vaccinia virus specific cytotoxicity against infected target cells was observed *in vitro*. Spleen lymphocytes from normal and immunized mice of the inbred strains C3H and DBA/2 were incubated with vaccinia virus-infected and non-infected ^{51}Cr-labeled mastocytoma P-815-X2 cells and L-929 fibroblasts, which were used as targets. Cytotoxic lymphocytes could be isolated from the mice as early as 2 days after infection with vaccinia virus. The highest cytotoxic effect was obtained with lymphocytes taken 6 days after infection. The degree of lysis was correlated with the ratio of immune lymphocytes to target cells. Specific blocking of target cell lysis resulted after addition of anti-vaccinia antibody from different sources. The effector cells could be characterized as T cells by elimination of macrophages and B cells. Target cell killing was only possible in a syngeneic system; allogeneic infected target cells were not lysed significantly.

[I 974]

1. Introduction

Cell-mediated immune mechanisms play an essential role in resistance to viral infections [1–4]. Lymphocyte transformation and migration inhibition tests have been adopted to evaluate the interactions between virus and lymphocytes. To prove cell-mediated immunity, the effect of T cells has to be demonstrated making it necessary to specify transformation tests with respect to the action of B and T cells. Furthermore, these tests give no evidence about the specific action of T cells

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Abbreviations. B cell: Antibody-forming cell precursor **CL:** Cytotoxic lymphocytes **CMI:** Cell-mediated immunity **FCS:** Fetal calf serum **LPS:** Lipopolysaccharide **MEM:** Eagle's minimum essential medium **PWM:** Pokeweed mitogen **PHA:** Phytohemagglutinin **T cell:** Thymus-derived cell **TCID:** Tissue culture infective dosis

on virus particles or virus-infected cells. Interactions between immune lymphocytes and virus-infected cells have been demonstrated *in vitro* using different viruses [5–15]. In this study we established an *in vitro* system to show cytotoxic actions of spleen cells from vaccinia virus-infected mice against vaccinia-infected target cells. We investigated: (a) the kinetics of *in vivo* production of cytotoxic lymphocytes (CL), (b) the cell type of the effector cell, (c) the interactions between antibodies and CL, (d) the virus specificity and the target cell specificity of the *in vitro* reaction.

2. Materials and methods

2.1. Viruses and animals used

Vaccinia virus strain WR was propagated in Vero cells (Green African monkey kidney) cultivated in Eagle's medium supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 10 % inactivated calf serum. Stock virus contained 10^6 $TCID_{50}$/ml. Herpes simplex virus type I was grown in Vero cells; the stock virus was equilibrated to 10^6 $TCID_{50}$/ml. Titration of virus was performed in Vero cells using the microtiter system (Greiner, Nürtingen, No 220 ART) with eight wells for every logarithmic dilution step. Reading was performed according to the method of Reed and Muench. Mice of the inbred strains C3H/TIF and DBA/2 (purchased from G.L. Bomholtgård, Ry Denmark) were used throughout the study.

2.2. Lymphoid cell suspensions

Lymphocytes were obtained from the mouse spleens at different times after injection of 1–2 ml 10^5–10^6 $TCID_{50}$/ml of vaccinia virus, herpes simplex virus or Eagle's medium. Cell suspensions from spleens were obtained by the following procedure: spleens from five mice were aseptically removed, homogenized by hand with one gentle stroke in a 15 ml glass homogenizer (Tenbroeck) containing 10 ml Eagle's medium supplemented with 10 % fetal calf serum, 1 % L-glutamin (200 mmoles), 1 % 50-fold concentrated amino acids (Microbiological Assoc., Bethesda, Md., No 13-606), 100 IU/ml penicillin and 100 µg/ml streptomycin. The suspension was transferred to a tube, underlayed with calf serum and incubated for 5 min at 4 °C to remove spleen debris and clumped cells. The supernatant was removed and underlayed again with serum and the cells were pelleted by centrifugation for 10 min with 250 x g at 4 °C. The supernatant containing smooth debris was discarded. The cells were resuspended and viability was tested by trypan blue exclusion. Depletion of erythrocytes was performed by Ficoll-Isopaque discontinous gradient centrifugation according to the method of Boyum [16]. Adherent cells were removed by passing the spleen cells through a column of siliconed glass beads according to the method of Shortman et al. [17]. The efferent cell population was free of macrophages as was confirmed by Giemsa staining.

2.3. B cell elimination

Immunoglobulin-bearing cells were separated by adherent properties. Spleen cell suspensions were first incubated in nylon wool columns for 45 min at 37 °C and then washed slowly with warm medium according to the method of Julius et al. [18]. The decrease of the percentage of Ig-bearing cells was controlled by direct immunofluorescence staining with fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig (Microbiological Assoc., No 51-792). The amount of labeled cells of the effluent cell fraction was 1 %, while the unfractionated cell suspensions contained 45–50 % Ig-bearing cells. Elimination of B cells was also tested by the capability of lymphocytes to respond to mitogens [19]. The response to phytohemagglutinin (PHA) (Wellcome, Buckingham, Kent) pokeweed mitogen (PWM) (Grand Island Biol. Co., Grand Island, N.Y. Cat. No 536) and lipopolysaccharide W (LPS) (Difco, Detroit, Mich., Cat. No 3122-25) was studied by [^{3}H]thymidine incorporation. While there were no significant alterations of PHA transformation ratios, a significant reduction of transformation by PWM and LPA was obtained in the B cell-depleted cell fraction.

Elimination of B cells was performed by incubation with goat antiserum to mouse Ig (Gibco, BCL 1039). The serum was absorbed with C3H mouse thymocytes until there was no more cytotoxicity against thymocytes. 15 x 10^6 cells were incubated in 0.2 ml of antiserum 30 min in 37 °C, washed and incubated again with an equal volume of guinea pig complement absorbed with mouse liver powder.

2.4. T cell elimination

Anti-Θ serum from AKR mice was kindly provided by Dr. Röllinghoff, Mainz. 15 x 10^6 spleen cells were incubated in 0.2 ml anti-Θ serum for 30 min at 37 °C. Controls were run with normal mouse serum. The cells were washed and resuspended in 0.2 ml guinea pig complement absorbed with mouse liver powder and agarose [20]. After incubation for 30 min at 37 °C the cells were washed and resuspended to the desired concentration. The viability of the cells was tested by the trypan blue exclusion test.

2.5. Anti-vaccinia serum

Hyperimmune serum was obtained from sensitized rabbits injected intracutaneously with 5 ml 10^6 $TCID_{50}$/ml followed 6–10 weeks later by booster intravenous injections of the same dosage. Syngeneic hyperimmune serum was obtained from mice after three intraperitoneal injections of 2 ml 10^6 $TCID_{50}$/ml given at two week intervals. The animals were bled one week after the last injection. Normal serum was obtained from noninfected animals. All sera were inactivated and stored at −70 °C.

2.6. Target cells

L-929 cells, kindly provided by Dr. Lehmann-Grube, Hamburg, were grown as monolayers in MEM containing 10 % FCS and 100 µg/ml of penicillin and streptomycin in Roux bottles. Mastocytoma P-815-X2 suspension cultures were grown in 10 cm Petri dishes containing Dulbecco's fortified Eagle's medium (Grand Island Biological Co.) with 10 % FCS and antibiotics, on a rocker platform (Bellco Glass Co, Vineland, N.Y.) in a humidified 8 % CO_2-atmosphere.

2.7. Infection of target cells

Monolayers of L-929 cells were trypsinized, suspended in fresh medium and plated in 0.1 ml volumes in sterile flat bottom microtest plates (Greiner, Nürtingen No 220 ART) to reach a final concentration of 5 x 10^4 cells/well. Five hours later the medium was removed and the cells were incubated with 0.2 ml virus suspension containing 5 x 10^5 $TCID_{50}$/0.2 ml medium. Two hours later all the supernatants were discarded and fresh medium was added. Suspensions of mastocytoma P-815-X2 cells were infected by incubating the cells in a medium containing 10 $TCID_{50}$ of virus per mastocytoma cell under constant rocking. After 2 h the cells were washed and resuspended in fresh medium.

Five times 10^6 infected or noninfected mastocytoma cells were suspended in 0.2 ml medium and 100 μCi ^{51}Cr (sodium-chromat, Amersham, Buchler, Braunschweig No CJS1P, spec. activity 100–200 mCi/mg Cr) was added. After 2 h incubation the cells were washed three times, resuspended to the required concentration and dispensed in microtiter wells to a final concentration of 5 x 10^4 cells/well. Infected or noninfected L-929 monolayers were labeled by adding 1 μCi ^{51}Cr to each well. After 2 h incubation the monolayers were gently washed three times.

2.8. Cytotoxic assay

The assay is a modification of the technique described by Brunner et al. [21]. Lymphocytes and target cells were incubated in sterile flat bottom microtiter plates with a volume of 0.25 ml per well at 37 °C in an atmosphere of 8 % CO_2 and 92 % air. Lymphocytes from infected and control mice were added to the ^{51}Cr-labeled normal or infected target cells usually reaching a final ratio of 100 spleen cells to 1 target cell in a volume of 0.2 ml. Routinely, 5 x 10^6 spleen cells were added to 5 x 10^4 target cells. If the number of immune spleen cells was less than 5 x 10^6, the requisite number of cells from noninfected animals was added to give the required count of 5 x 10^6 lymphocytes.

Following incubation for 4–20 h the microplates were centrifuged at 1000 rpm for 5 min. 0.1 ml of cell-free supernatant was gently removed and the residual 0.1 ml volume was harvested by vigorous pipetting to suspend the adherent cells. The volumes containing cell-free supernatant or supernatant with cells were counted and ^{51}Cr release in the whole supernatant was calculated using the formula:

$$\text{Release} = \frac{\text{counts in supernatants} - \text{background counts}}{\text{counts in whole supernatant + cells} - \text{background counts}} \times 100$$

The percentage of specific cytolysis of infected target cells was calculated by subtracting from the percentage ^{51}Cr released in the presence of immune spleen cells that percentage of ^{51}Cr released by the same number of cells from noninfected animals.

3. Results

3.1. Infection conditions

The infection of mastocytoma and L-929 cells with 10 $TCID_{50}$/cell vaccinia virus induces cytolysis. In the L cells, which were grown as monolayers, the infection led to the appearance of round cells after 4 h, followed by aggregation of cells after 9–10 h and later on, to reduced adherence of the infected cells to the surface. Loss of the ^{51}Cr label was modest and generally did not exceed 5–25 % after 30 h, while noninfected L-929 cells showed spontaneous release between 10–20 % (Table 1). A significant increase of infectivity titer was observed after 30 h. The mastocytoma P-815-X2 suspension cultures had a similar spontaneous ^{51}Cr release after infection (Table 5). The infected cells could be used 2 h after infection, but often higher percentage of specific cell cytolysis by immune lymphocytes could be obtained with cells infected for a longer period. Twelve hour infected cells were usually utilized as targets.

The development of early virus-specific antigens after vaccinia virus infection has already been described [22]. We could observe specific surface immunofluorescence in infected L-929 or mastocytoma P-815-X2 cells, beginning 2 h after infection, using human anti-vaccinia hyperimmune sera. Fluorescence was more pronounced and visible on a higher percentage of cells in later stages of infection, reaching a percentage of 70–80 % of labeled cells.

3.2. Effects of sensitized lymphocytes on infected target cells

C3H/TIF mice were infected intraperitoneally with 2 ml Eagle's medium containing 10^6 $TCID_{50}$/ml vaccinia virus. Control animals received virus-free medium. Six days later the mice were sacrificed, the spleens of 5 mice from each group were pooled and spleen cell suspensions were prepared. The cells were incubated for 20 h with ^{51}Cr-labeled, infected and noninfected L-929 cells and the ^{51}Cr release was calculated. Incubation of spleen cells from sensitized mice with vaccinia virus-infected target cells led to significant chromium release, while noninfected targets remained unaffected. Control spleen cells did not relevantly affect the target cells (Table 1).

Table 1. Cytotoxic effect[a] of C3H lymphocytes against vaccinia virus-infected L-929 cells

Lymphocytes[b]	Target cells: Infected[c]	Target cells: Noninfected
Vaccinia virus-infected spleen	82.4 ± 3.4[d]	36.6 ± 0.3
Normal spleen	28.2 ± 2.4	30.0 ± 1.7
Nil	24.5 ± 0.7	17.5 ± 0.8

a) Mean % ^{51}Cr release ± standard error of the mean from groups of 5 wells.
b) Lymphocytes from a pool of 4–6 mice, purified by Ficoll-Isopaque gradient centrifugation. Mice were injected 6 days previously with 1 ml 1 x 10^6 $TCID_{50}$/ml vaccinia virus i.p. Ratio lymphocytes: targets = 100:1.
c) 5 x 10^4 L-929 cells/well infected 12 h previously with 5 x 10^5 $TCID_{50}$/0.2 ml of vaccinia virus for 2 h at 37 °C, then incubated in complete medium.
d) Significantly greater ^{51}Cr release from virus-infected L-929 cells by immune lymphocytes compared to normal lymphocytes ($P < 0.001$); significantly greater ^{51}Cr release by immune lymphocytes from infected L-929 cells compared to noninfected L-929 cells ($P < 0.001$).

3.3. Virus specificity of infected target cell lysis

C3H mice were injected with 1 x 10^7 viable BCG organisms (Behring-Werke, Marburg), 1 ml MEM containing 1 x 10^6 $TCID_{50}$/ml herpes simplex virus type I, 1 ml MEM containing 1 x 10^6 $TCID_{50}$/ml vaccinia virus or Eagle's medium alone. The spleen cells were harvested 6 days later and incubated for 20 h with either vaccinia virus-infected or noninfected L-929 cells. Only cells from vaccinia virus-sensitized animals were able to lyse the infected target cells in significant amounts (Table 2).

3.4. Time course of development of cytotoxic lymphocytes

C3H mice were injected with 2 ml containing 1 x $10^{5.4}$ $TCID_{50}$/ml vaccinia virus. Beginning 2 days after injection, groups of 5 infected or mock-infected animals were killed and the cytotoxicity test was performed. Occurrence of CL could be observed after 2 days, reaching a maximum after one week and declining in the second week after infection (Fig. 1). At day 12 only weak cytotoxicity remained.

Table 2. Specificity of vaccinia virus-infected target cell lysis[a)]

Lymphocytes[b)]	Infected L-929 cells
Normal spleen	6.0 ± 1.7
Vaccinia virus-infected spleen	58.2 ± 1.2[c)]
Herpes simplex virus-infected spleen	5.0 ± 2.0
BCG-infected spleen	12.5 ± 3.1

a) Mean % ^{51}Cr release ± SEM, 5 wells per group.
b) Lymphocytes from a pool of 6 mice/group. Mice were injected intraperitoneally 6 days previously with 1 ml 1 x 10^6 $TCID_{50}$/ml vaccinia virus, 1 ml 1 x 10^6 $TCID_{50}$/ml herpes simplex virus type I or 1 ml containing 10^7 viable BCG organisms. Ratio lymphocytes: infected target cells = 100:1.
c) Significant ^{51}Cr release ($P < 0.001$). No significant ^{51}Cr release in the other groups.

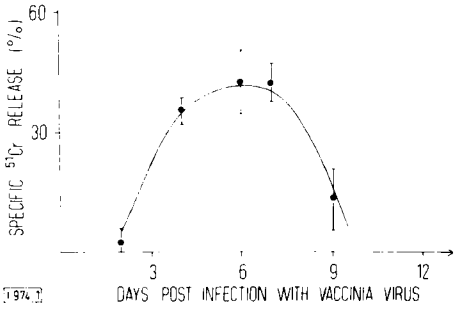

Figure 1. Time dependence on the appearance of cytotoxic effector cells *in vivo*. Percent specific ^{51}Cr release from mouse spleen cells harvested at different intervals after intraperitoneal injection of 2 ml $10^{5.4}$ $TCID_{50}$/ml vaccinia virus. Release of normal spleen cells was subtracted. Each point represents the mean of 5 wells ± 2 SEM, lymphocyte: target cell ratio = 100:1.

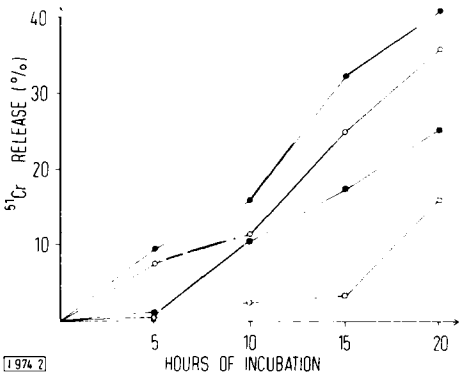

Figure 2. Kinetics of target cell lysis *in vitro*. Number of target cells (L-929, vaccinia infected): 5 x 10^4/well. Number of lymphocytes: 5 x 10^6/well. Mixtures of normal lymphocytes with lymphocytes from mice immunized 6 days previously; % ^{51}Cr release was measured at intervals of 5 h. Each point represents the mean of 5 wells. Percentage of immune lymphocytes: (□—□) 25, (■—■) 50, (○—○) 75, and (●—●) 100.

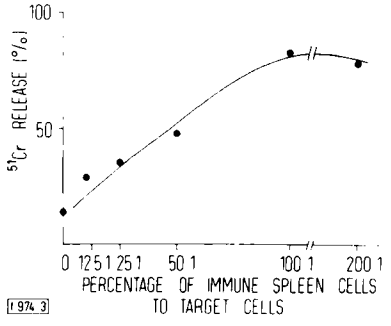

Figure 3. ^{51}Cr release with different lymphocytes to target cell ratios. Increasing numbers of lymphocytes from 6 day immunized mice were incubated with a constant number (5 x 10^4) of vaccinia virus-infected L-929 cells. Each point represents the mean of 5 wells.

3.5. *In vitro* kinetics of target cell lysis

The following experiment was performed using a constant ratio (100:1) of lymphocytes to target cells. Spleen lymphocytes were obtained from 6-day infected C3H mice and control animals. While the total amount of lymphocytes was kept constant, different percentages of lymphocytes from sensitized animals were mixed with normal lymphocytes and incubated with the target cells. At intervals of 5 h the reactions were stopped and the ^{51}Cr release was tested. Specific lysis could be observed as early as at 5 h incubation. The increase of ^{51}Cr release takes a nearly linear course when correlated with time. The time-dependent increase of ^{51}Cr release is consistent with the percentage of sensitized lymphocytes in the reaction (Fig. 2).

The ratio of 100 lymphocytes to 1 target cell seems to be the optimal concentration of effector cells to targets. Further additions of sensitized lymphocytes to a constant number of target cells exceeding the ratio 100:1 do not lead to an increase of chromium release (Fig. 3). In some experiments a slight decrease of ^{51}Cr release could be observed at ratios over 100:1.

3.6. Effect of anti-vaccinia antibodies on vaccinia virus-infected target cell lysis

Incubation of infected target cells with human anti-vaccinia antibody together with agarose-absorbed guinea pig complement for 20 h resulted in only slight cytolysis. Antibody or complement alone had no cytotoxic effect. The incubation of infected target cells, specific antibodies and sensitized lymphocytes together for 20 h led to a significant reduction of ^{51}Cr release. This blocking effect of antiserum could also be obtained using rabbit hyperimmune serum or syngeneic hyperimmune serum from C3H mice. Normal serum had no blocking activities (Table 3).

3.7. Characterization of the effector cell

In the *in vitro* system virus-specific cytolysis of vaccinia-infected target cells could be obtained with spleen cells and peritoneal exudate cells from vaccinia virus-infected animals, while normal cells had no effect. Immune spleen lymphocytes were incubated with anti-mouse Ig for 30 min, washed and reincubated with absorbed guinea pig complement. This procedure led to a 46 % killing of Ficoll-Isopaque purified cells. Spleen cell cytotoxicity was not found to be reduced significantly. Likewise the elimination of Ig-bearing cells by adherent properties in a nylon wool column had no effect on the cytolytic activity of the effluent cell fraction.

A glass bead column was used to eliminate macrophages. The removal of macrophages had no effect on target cell lysis. These results suggest that cells with adherent properties, including macrophages and B cells, and equally Ig-bearing cells such as B cells and other cells with surface adsorbed immunoglobulins, for example macrophages coated with

Table 3. Effect of anti-vaccinia antibodies on lymphocyte-mediated lysis[a] of vaccinia virus-infected L-929 target cells

Exp.	Lymphocytes	Serum	^{51}Cr release (%)
1	Immune spleen	Normal mouse	77.0 ± 1.3
		Vaccinia immune[b] mouse	35.0 ± 1.6[d]
	Immune spleen	Normal rabbit	66.5 ± 0.9
		Vaccinia immune[b] rabbit	34.5 ± 1.0[d]
2	Immune spleen	Normal human	77.3 ± 0.8
		Vaccinia immune[c] human	27.3 ± 1.4[d]
	Normal spleen	Normal human	31.0 ± 1.1
		Vaccinia immune human	26.3 ± 1.2
		Vaccinia immune human + guinea pig complement	30.6 ± 1.8

a) Lymphocytes from 6-day previously infected mice. Ratio lymphocytes: target cells 100:1, mean % ^{51}Cr release ± SEM, 3 wells per group;
b) Serum obtained after 3 injections of 1 ml 1 x 10^6 $TCID_{50}$/ml or 5 ml 1 x 10^6 $TCID_{50}$/ml (rabbit) vaccinia virus.
c) Hyperimmunglobulin (Vacciniabulin, Immuno^R) 48 h dialyzed against phosphate buffered saline, then diluted 1:5 in MEM.
d) Significant inhibition of ^{51}Cr release by immune mouse ($P < 0.001$), rabbit ($P < 0.001$) and human ($P < 0.001$) sera.

cytophilic antibodies, are not responsible for vaccinia virus-specific target cell killing.

After incubation of cells with anti-Θ serum and then with complement, 32 % of cells were dead, calculated by trypan blue exclusion. The treatment of spleen cell or peritoneal exudate cells with anti-Θ serum and complement resulted in significant reduction of target cell killing. Treatment with anti-Θ serum without complement is not followed by reduction of ^{51}Cr release (Table 4).

Table 4. Classification of the cytotoxic lymphocyte

Treatment of immune lymphocytes[b]	^{51}Cr release[a] from infected L-929 cells (%)
Untreated	52.0 ± 0.8
Nylon wool column adsorption	53.0 ± 2.0
Glass bead column adsorption	48.9 ± 1.6
Anti-mouse Ig + C	41.6 ± 1.2
Anti-Θ serum + C	17.2 ± 2.3[c]
Anti-Θ serum without C	45.5 ± 0.9

a) Mean % ^{51}Cr release, SEM from groups of 5 wells.
b) Lymphocyte from 6-day infected mice, lymphocyte: target cell ratio = 50:1.
c) Significant reduction ($P < 0.001$) of target cell lysis after pretreatment of lymphocytes with anti-Θ serum and complement.

3.8. Effect of target cell specificity on virus-specific lymphocyte cytotoxicity

Sensitized lymphocytes from C3H mice were able to kill vaccinia-infected L-929 cells; the noninfected cells usually were not affected. When lymphocytes from DBA/2 mice were used, the vaccinia-infected L-929 cells were not killed by the normal or the immune lymphocytes. Likewise the immune lymphocytes from C3H mice no longer had cytolytic activities on vaccinia-infected mastocytoma P-815-X2 target cells. Immune cells from C57BL/6J mice had no significant effects on vaccinia infected L-929 cells or vaccinia-infected mastocytoma P-815-X2 cells (unpublished data). When a cross-reaction was performed using cells from normal and infected C3H and DBA/2 mice against infected and noninfected L-929 and mastocytoma P-815-X2 target cells. C3H lymphocytes only lysed infected L-929 cells, while DBA/2 lymphocytes only lysed the infected mastocytoma P-815-X2 cells (Table 5).

Since C3H mice and L-929 cells share the same H-2^k antigen and DBA/2 mice have the same H-2^d antigen as do mastocytoma P-815-X2 cells, it could be concluded that vaccinia virus-specific lymphocyte cytotoxicity for target cells is dependent on transplantation antigen compatibilities.

Table 5. Percent ^{51}Cr release[a] from vaccinia virus-infected and noninfected target cells by CL from normal and vaccinia virus-infected mice[b]

Spleen lymphocytes	L-929		Mastocytoma P-815-X2	
	Infected	Noninfected	Infected	Noninfected
	^{51}Cr release (%)			
None	24.7 ± 1.1	13.1 ± 2.3	24.1 ± 1.3	21.0 ± 1.6
C3H (H-2^k)				
immune	77.0 ± 1.2[c]	33.5 ± 0.9	13.0 ± 1.5	5.6 ± 0.4
normal	28.3 ± 2.7	30.8 ± 0.7	10.4 ± 1.2	1.8 ± 0.4
DBA/2 (H-2^d)				
immune	19.2 ± 2.8	32.2 ± 1.8	60.6 ± 1.6[c]	2.0 ± 0.3
normal	16.0 ± 3.2	20.2 ± 3.1	12.4 ± 1.0	2.4 ± 0.5

a) Mean of 5 wells ± SEM in each group.
b) Mice were injected intraperitoneally 6 days previously with 1 ml 10^6 $TCID_{50}$/ml of vaccinia virus.
c) Significantly greater ^{51}Cr release ($P < 0.001$) of immune lymphocytes against syngeneic infected target cells compared to the action of normal cells, or to the action of immune lymphocytes against allogeneic infected and noninfected target cells.

4. Discussion

Most systems in which specific killing of virus-infected target cells by immune lymphocytes has been described, work with viruses which themselves have no or only slight cytolytic effects on the target cells. Recently, virus-specific cytotoxicity could also be demonstrated in an acute virus infection which leads to virus-induced target cell destruction [14]. Vaccinia virus infection eventually results in target cell destruction, so that the incubation time for the *in vitro* experiment is limited. In spite of this restriction it is a good model to study the *in vitro* actions of immune effector cells.

The time course of development of CL in our experiments was similar to other experimental systems investigating cytotoxic effects on cells infected with different viruses [12, 15]. One can suggest that the appearance and disappearance of cytotoxic lymphocytes take a regular course, but there is no information as to what happens to this effector cell population after the decrease of cytotoxicity two weeks after infection. It is not clear if these cells are identical to those that show antigen-specific blast transformation and release of factors. The appearance of lymphocytes which develop specific blast transformation to vaccinia virus seems to be delayed com-

pared with the appearance of cytotoxic cells. In transformation experiments with lymphocytes from vaccinated persons no booster effect in blast transformation after revaccination could be observed [23], while there was an increase of interferon production [24]. Experiments are under study investigating the possibility of reactivating cytotoxic lymphocytes *in vivo* after reinfection.

The kinetic studies show that the rate of target cell lysis is augmented constantly with time and with increasing numbers of CL. The results represented here are virus-specific; CL from animals infected with herpes simplex virus type I or viable BCG organisms have no effect on vaccinia virus-infected target cells. A second argument for virus specificity is the blocking effect of anti-vaccinia antibody. Blocking could be achieved with antibodies from different species. It was not possible to show a blocking effect when the ectromelia virus was used in the same system. The possibility of recognizing antigens of different complexity by B and T cells [25], competition of T cells and antibodies for the receptor or merely insufficient concentration of antibodies were discussed. We suggest that inadequate concentrations of antibodies may have led to negative results, since dilution of antibody to 1:25 resulted in only minimal inhibition. On the other hand one has to take into account that antibodies which are capable of neutralizing the infective virus may differ in their activity against virus-specific surface antigens.

It has been demonstrated that antibodies can be raised which are directed only against virus-specific surface antigens or viral structural antigens of vaccinia virus [26]. It has, however, to be elucidated if antibodies, which are directed solely against viral structural antigens, are able to block the action of CL.

The studies with purified T cells after the elimination of B cells and macrophages using adherent properties, as well as the effects of anti-Ig antibodies and anti-Θ serum, lead to the suggestion that the T cell alone is the effector cell which is able to lyse the infected target cell, in the absence of B cells, antibodies, complement or other soluble factors. The results are consistent with the findings of other investigations in which the cell type of CL against virus-infected cells [13, 14, 27] or cells differing by transplantation antigens or tumor-associated transplantation antigens could be characterized as a T cell [28, 29].

It seems obvious that CL play a role in host resistance to viral infections, but the importance of this effect remains to be defined. It has been shown recently that in an acute infection with sindbis virus, CL have no effect on virus clearance [14]. Virus clearance could be attributed to the action of macrophages which may act in an activated state or be "armed" by immune T cells [30] with or without the help of antibodies or lymphocytes [31]. In the vaccinia system we could show that clearance of virus *in vitro* is possible without the cytolysis by CL (manuscript in preparation).

In our experimental system CL from C3H mice could only kill infected L cells but not infected or noninfected mastocytoma P815-X2 cells. The action of CL from DBA/2 mice was also specific; they had cytolytic effects only on infected mastocytoma cells. In a recent investigation it has been observed [15] that in the lymphocytic choriomeningitis system, target cell lysis was restricted to syngeneic or semiallogeneic systems. Since LCM is a budding virus it seems possible that this effect was caused by this type of virus maturation, followed by alteration of H-2 antigen concentration on the surface of infected cells [32]. The H-2 antigen-specific restriction of cytolysis in the vaccinia system suggests that this restriction is caused by a more general phenomenon. This is supported by the data of Shearer [33], showing cell-mediated cytotoxicity to trinitrophenyl-modified lymphocytes in a syngeneic system. In another pox virus system using ectromelia virus, different CL activities on various-infected cells were interpreted as different expressions of viral surface antigens on the target cells [13]. However, if one correlates the results of specific target cell destruction with H-2 antigen compatibility, there is a decrease of cytolytic effects consistent with increasing antigenic heterogenicity. Secondly, the infected mastocytoma cell was, in our system, just as lysable as the L cells under the prerequisite that the syngeneic cytolytic effector cell was used. Other investigators communicated virus-specific lysis of heterologous infected target cells [5, 10, 11]. For these systems the T cell specificity of the reaction has to be elucidated to exclude antibody-dependent activities [34–37]. On the other hand, one could also suppose that high concentrations of virus-specific antigens on cell surfaces can be recognized in spite of foreign transplantation antigen structures.

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5. References

1 Von Pirquet, C.E., *Arch. Intern. Med.* 1911. *7*: 259.

2 Glasgow, L.A., *Arch. Intern. Med.* 1970. *126*: 125.

3 Good, R.A. and Varco, R.L., *Lancet* 1955. *75*: 245.

4 Fulginiti, V.A., in Kagan, B.M. and Stielum, E.R. (Eds.), *Immunologic Incompetence*, Yearbook Publ., Chicago 1971, p. 245.

5 Speel, L.F., Osborn, J.E. and Walker, D.L., *J. Immunol.* 1968. *101*: 409.

6 Lundstedt, C., *Acta Pathol. Microbiol. Scand.* 1969. *75*: 139.

7 Oldstone, M.B. and Dixon, F.I., *Virology* 1970. *42*: 805.

8 Marker, O. and Volkert, M., *J. Exp. Med.* 1973. *137*: 151.

9 Profitt, M.R., Hirsch, M.S. and Black, P.H., *J. Immunol.* 1973. *110*: 1183.

10 Steele, R.W., Hensen, S.A., Vincent, M.M., Fucillo, D.A. and Bellanti, J.A., *J. Immunol.* 1973. *110*: 1502.

11 Labowskie, R.A., Edelman, R., Rustigian, R. and Bellanti, J.A., *J. Infec. Dis.* 1974. *129*: 233.

12 Gardner, I., Bowern, N.A. and Blanden, R.V., *Eur. J. Immunol.* 1974. *4*: 63.

13 Gardner, I., Bowern, N.A. and Blanden, R.V., *Eur. J. Immunol.* 1974. *4*: 68.

14 McFarland, H.F., *J. Immunol.* 1974. *113*: 173.

15 Zinkernagel, R.M. and Doherty, P.C., *Nature* 1974. *248*: 701.

16 Boyum, A., *Scand. Clin. Lab. Invest.* 1968. Suppl. 21. *97*: 77.

17 Shortman, K., Williams, N., Jackson, H., Russell, P., Byrt, P. and Diener, E., *J. Cell. Biol.* 1971. *48*: 566.

18 Julius, M.H., Simpson, E. and Herzenberg, L.A., *Eur. J. Immunol.* 1973. *3*: 645.

19 Janossy, G. and Greaves, M.F., *Clin. Exp. Immunol.* 1971. *9*: 483.

20 Cohen, A. and Schlesinger, M., *Transplantation* 1970. *10*: 130.

21 Brunner, K.T., Mauel, J., Cerottini, J.-C. and Chapuis, B., *J. Immunol.* 1968. *14*: 181.

22 Ueda, Y., Tagaya, I., Amano, H. and Ho, M., *Virology* 1972. *49*: 794.

23 Koszinowski, U., Volkmann, B. and Thomssen, R., *Z. Immunitätsforsch. Exp. Ther.* 1975, in press.

24 Epstein, L.B., Stevens, D.S. and Merigan, T.C., *Proc. Nat. Acad. Sci. US* 1972. *69*: 2632.

25 Alkan, S.S., Williams, E.B., Nitecki, D.E. and Goodman, J.E., *J. Exp. Med.* 1972. *135*: 1228.

26 Ueda, Y. and Tagaya, I., *J. Exp. Med.* 1973. *138*: 1033.

27 Zinkernagel, R.M. and Doherty, P.C., *J. Exp. Med.* 1973. *138*: 1266.

28 Wagner, H. and Röllinghoff, M., *J. Exp. Med.* 1973. *138*: 1.

29 Wagner, H., *J. Exp. Med.* 1973. *138*: 1379.

30 Evans, R. and Alexander, P., *Nature* 1972. *236*: 168.

31 Lohmann-Mathes, M.-L., Schipper, H. and Fischer, H., *Eur. J. Immunol.* 1972. *2*: 45.

32 Hecht, T.T. and Summers, D.F., *J. Virol.* 1972. *10*: 578.

33 Shearer, G.M., *Eur. J. Immunol.* 1974. *4*: 527.

34 Perlmann, P. and Perlmann, H., *Cell. Immunol.* 1970. *1*: 300.

35 Perlmann, P., Perlmann, H. and Wigzell, H., *Transplant. Rev.* 1972. *13*: 91.

36 Calder, E.A., Penhale, W.J., Barnes, E.W. and Irvine, W.J., *Clin. Exp. Immunol.* 1973. *14*: 19.

37 Schirrmacher, V., Rubin, B., Pross, H. and Wigzell, H., *J. Exp. Med.* 1974. *139*: 93.

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Early detection of potentially lethal events in T cell-mediated cytolysis*

The short-term kinetics of the interaction between mouse cytolytic T lymphocytes (CTL) and ^{51}Cr-labeled target cells was investigated. It was found that addition of EDTA to mixtures of CTL and target cells instantaneously blocked *de novo* lytic interactions, but did not inhibit the release of ^{51}Cr from already damaged target cells. Using this information, a modified cytolytic assay was developed. By applying this assay to highly active CTL populations generated in secondary mixed leukocyte cultures, it was possible to detect appreciable target cell damage as early as 30 seconds after exposure to CTL. Quantitative studies demonstrated linear relationships between cytolysis and time and between rate of cytolysis and cell number under these assay conditions.

1. Introduction

The specific *in vitro* destruction of ^{51}Cr-labeled target cells by cytolytic thymus-derived lymphocytes (CTL) is a well-characterized model system in which to study the efferent phase of cell-mediated immunity [1]. At the present time, however, studies of the mechanism of CTL-mediated cytolysis at the molecular level are restricted by the relatively long duration (3–18 h) of commonly used assay systems. In order to circumvent this problem, we have modified the cytolytic assay so as to restrict the interaction between CTL and target cells to very short time periods. The rationale for this modification is based on the earlier observation that the addition of ethylenediaminetetraacetic acid (EDTA) to mixtures of CTL and target cells resulted in complete inhibition of ^{51}Cr release if EDTA was added at the beginning of the assay period, but only partial inhibition when added at later times [2, 3]. In view of the recent demonstration that ^{51}Cr release may continue for several hours following inactivation of CTL by antibody and complement [4] or by heating to 45 °C [5], a likely interpretation of the earlier results is that EDTA prevents the *de novo* initiation of target cell damage, but has no effect on the release of ^{51}Cr from already damaged target cells.

In the present study, the minimal duration of cell contact necessary to initiate lethal target cell damage has been estimated by making use of this property of EDTA. In particular, by applying this analysis to highly active CTL populations generated in secondary mixed leukocyte cultures (MLC-Imm) [6], we have been able to detect appreciable target cell damage as early as 30 sec after exposure to CTL at appropriate temperatures. Furthermore, the rate of target cell damage has been found to proceed linearly with time under these experimental conditions. The significance of these findings for the utilization and interpretation of ^{51}Cr release assays will be discussed.

2. Materials and methods

2.1. Mice

Adult female mice of the inbred strains C57BL/6 and DBA/2 were supplied by the animal colony maintained at the Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland. Original breeding pairs were obtained from the Jackson Laboratories, Bar Harbor, Maine.

2.2. Generation of effector cells

Cytolytic thymus-derived lymphocytes (CTL) were generated in mixed leukocyte cultures (MLC) using C57BL/6 spleen cells as responding cells and irradiated (1000 rads) DBA/2 spleen cells as stimulating cells. A detailed description of the culture conditions has been published elsewhere [6]. In some

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Abbreviations: CTL: Cytolytic thymus-derived lymphocytes **MLC:** Mixed leukocyte culture **MLC-Imm:** Secondary mixed leukocyte culture