COLD SPRING HARBOR SYMPOSIA ON QUANTITATIVE BIOLOGY

VOLUME XLI—PART 2

COLD SPRING HARBOR SYMPOSIA ON QUANTITATIVE BIOLOGY

VOLUME XLI

Origins of Lymphocyte Diversity

COLD SPRING HARBOR LABORATORY

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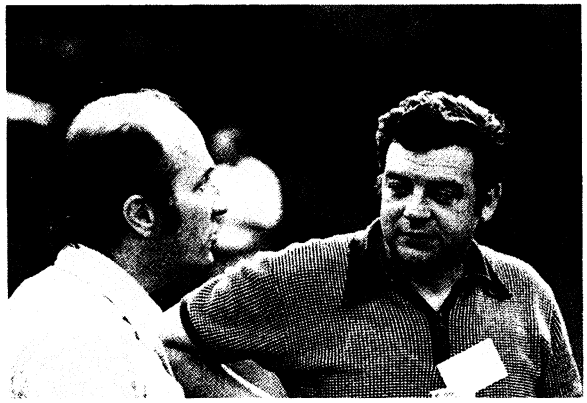

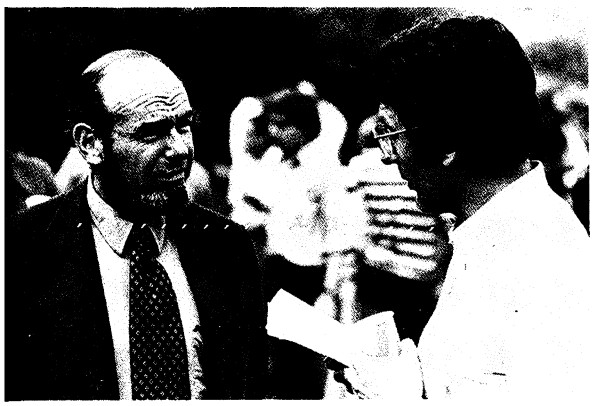


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Summary

Recognition of Alterations Induced by Early Vaccinia Surface Antigens and Dependence of Virus-specific Lysis on H-2 Antigen Concentration on Target Cells

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Mice generate cytolytic T lymphocytes (CTL) after sensitization with infective virus (Zinkernagel and Doherty 1974; Gardner et al. 1975; Koszinowski and Thomssen 1975; Ertl and Koszinowski 1976). CTL lyse virus-infected target cells in vitro. The reaction is specific for the sensitizing virus; there is no cross-reactivity against cells infected with viruses of another group. In addition to virus specificity, H-2 homology of antigenic products of the K or D region is required for specific lysis (Zinkernagel and Doherty 1975). The same requirements have been found in the effector phase against chemically modified cells (Shearer 1974; Shearer et al. 1975; Forman 1975; Rehn et al. 1976) and cells bearing the male-specific antigen (H-Y) (Gordon et al. 1975) as well as in the reactivity against minor histocompatibility antigens (Bevan 1975). The question arose whether this restriction is due to a dual recognition process of syngenic H-2 structures and viral antigens or to a single T-cell receptor which recognizes a virus-specific altered H-2 antigen. Recent experiments with radiation chimeras favor the second possibility since reaction against "modified" alloantigens is possible provided the CTL is tolerant to the alloantigen which is present during the sensitization phase (Pfizenmaier et al. 1976).

It must be considered, however, that determination of ^{51}Cr-release in certain combinations reflects only the requirements of the lethal-hit stage (Golstein and Smith 1976). The inference as to recognition requirements can only be drawn indirectly from cytolysis data. Requirements for recognition can be tested by specific absorption on monolayers (Wekerle et al. 1972; Golstein et al. 1972). Data presented here show that absorption is possible only on monolayers bearing "modified" H-2 determinants present during the sensitization phase. Another question is whether CTL can lyse virus-specific infected cells which lack H-2 antigenic determinants. To investigate the relationships between H-2 antigenic concentration and degree of virus-specific lysis, infected target cells treated with proteolytic enzymes were tested. In a third approach, we looked for the minimal changes that must be induced on cells by the vaccinia virus infection to sensitize CTL in vivo and to enable cytolysis in vitro. Our data demonstrate that induction of early vaccinia virus surface antigens is sufficient for sensitization and specific lysis.

MATERIALS AND METHODS

Mice. Mice of the inbred strains C3H (H-2^k), DBA/2 (H-2^d), and C57BL/6J (H-2^b) at the age of 6–10 weeks were used throughout. These were purchased from Bomholtgard, Denmark.

Viruses and sensitization. Stocks of vaccinia virus strains WR and Elstree were grown in VERO *(Cercopithecus aethiops)* kidney cells. Virus titrations were performed on the same cells. Stock solutions of vaccinia virus strains WR and Elstree (Lister strain) contained 1×10^6 $TCID_{50}$ (tissue-culture infective dose) per milliliter. Strain DIs, an attenuated vaccinia virus mutant which derived from the Dairen 1 (DIE) strain (Tagaya et al. 1961), was provided by Dr. Ueda, Tokyo. DIs was propagated in 12-day-old fertile eggs at 35–36°C for 2 days. Titrations were done on primary chicken fibroblasts (Tagaya et al. 1974). DIs was used in a concentration of 1×10^5 $TCID_{50}$ per milliliter. Purification of strain WR was performed according to the method of Joklik (1962). Inactivation of vaccinia virus WR was obtained by incubation at 56°C for 120 minutes, three times interrupted by a short sonication procedure.

LCM virus strain WE-3, kindly provided by Dr. Lehmann-Grube, Hamburg, was propagated in L929 cells.

Virus of vesicular stomatitis (VSV) strain Indiana was propagated in VERO cells; stock solutions contained 10^6 $TCID_{50}$ per milliliter. Mice were sensitized by intraperitoneal injection of the 1-ml virus suspension. Spleen cells were harvested 6 days later.

Antisera. Antiserum to strain WR (No. 8) was obtained from rabbits immunized with WR strain vaccinia virus. The animals were injected intradermally, and, after a period of 2 weeks, three booster injections were given at intervals of 10 days. Sera were inactivated and stored at −70°C. The cytolysis titer of the sera measured in a ^{51}Cr-release assay, using infected L929 cells as targets

and guinea pig serum as complement source, was about 1024–2048. An anticomplementary activity of the sera was demonstrable to a dilution of about 1:8.

Antiserum to late vaccinia virus surface antigens (anti-LS): serum No. 8 was extensively absorbed with DIs-infected primary chicken fibroblasts. Antiserum to early vaccinia virus surface antigens (anti-ES): rabbit serum against early vaccinia virus surface antigens was prepared by injection of crude soluble early antigens of DIs-infected rabbit kidney cells into rabbits (Ueda et al. 1972; Ueda and Tagaya 1973). Antiserum to structural antigens of the virion (anti-VA) was obtained from rabbits injected with purified inactivated vaccinia virus. One optical density unit (ODU) (Joklik 1962), containing about 64 μg viral protein, was injected intramuscularly, and a booster injection of the same dose was given 14 days later. Neutralizing antibody titers were determined by 80% plaque reduction in VERO cell cultures.

Anti-H-2 serum was obtained from D. J. G. Ray, Transplantation and Immunology Branch, NIAID, NIH Bethesda, Maryland. We used charge 3b(Snell 1974), raised in recipient-donor strains (C3H-H-2^0 × 129) anti-C3H, genotypes (H-$2^{d/k}$ × H-2^b) anti-H-2^k. The serum is directed mainly against specificities coded by H-2 K genes. No cross-reactivity with other private H-2 specificities could be observed by immunofluorescence or complement-dependent antibody-mediated cytolysis.

Demonstration of viral surface and intracellular antigens. Indirect immunofluorescence was performed as reported previously (Koszinowski and Ertl 1975). Demonstration of DNA synthesis in vaccinia-virus-infected cells was performed with DAPI (4,6-diamidino-2-phenylindol) (Russell et al. 1975).

Early and late surface antigens of cells infected with vaccinia virus were studied by mixed hemagglutination technique (Ito and Barron 1972) and by cytolytic antibody assay.

Target cells: L929 cells (haplotype H-2^k) and mastocytoma P-815-X2 cells were cultivated in Eagle's MEM supplemented with 10% inactivated calf serum and 100 μg/ml streptomycin and penicillin (1% SP). For infection of target cells, about 5×10^6 to 1×10^7 L929 cells growing as monolayer cells in glass bottles were washed with fresh serum-free medium and then incubated with 10–15 ml virus suspension on a rocker platform at 37°C. After 2 hours, the virus dilution was discarded and fresh serum-free medium was added. The cells were used 3–12 hours later, depending on virus and test conditions.

Enzymatic treatment: The following enzymes were used: (1) papain, water-soluble, 3.5 mAnson units per milligram (Merck, Darmstadt, W. Germany, lot 7144); and (2) neuraminidase from vibrio comma (VCN), 500 neuraminidase units per milliliter (Behringwerke, Marburg/Lahn, W. Germany). The enzymes were dissolved and diluted in serum-free MEM.

Cells prepared for enzymatic treatment were washed twice with serum-free medium and diluted to a concentration of 2.5×10^6 cells/ml. The enzymatic treatment was performed at 37°C, pH 6.5–7.0, with intermittent shaking for 20 (papain) or 30 minutes (VCN). Treatment with papain was carried out in the presence of 0.01 M cysteine (L(+)-cysteine chloride, Merck, lot 2839). Following enzymatic treatment, the cells were washed three times with MEM supplemented with 20% fetal calf serum (FCS).

Cells were counted and viability was determined by the trypan blue exclusion test. With the enzyme concentrations used, viability did not fall below 70–80%. The cells were stored before use in Eagle's MEM supplemented with 20% FCS. For virus-specific cytotoxic assays, carried out 4 hours after enzymatic treatment, cells were labeled 1 hour before with 100 μCi ^{51}Cr (sodium chromate; Amersham, Buchler, Braunschweig, W. Germany; No. CJS1P, specific activity 100–200 mCi/mg Cr) per 5×10^6 cells. For allogenic cell-mediated cytolysis (CMC), cells were labeled before enzymatic treatment and used as targets immediately after treatment.

Sensitization to alloantigens. Mitomycin-C-treated stimulator cells, 1×10^6, were incubated with 4×10^6 responder cells in 3 ml Dulbecco's modified medium supplemented with 5% FCS and 100 μg/ml penicillin and streptomycin in a CO_2 atmosphere at 37°C. Allogenic spleen cells or tumor cells were used as stimulator cells. Five or six days later, the cells were harvested, and their effect on the appropriate ^{51}Cr-labeled target cells was tested in a 5–10-hour assay. The ratio of attacker to target cells ranged between 20:1 and 50:1.

Adsorption of effector cells on monolayers. Mouse peritoneal exudate cells were harvested after thioglycolate stimulation and plated in plastic petri dishes (100-mm diameter, Greiner, Nürtingen, W. Germany). Confluent monolayers were infected 24 hours later. The medium containing virus was replaced by fresh medium after an adsorption time of 1 hour. About 6–12 hours later, monolayers were washed carefully and used for lymphocyte absorption. Spleen-cell suspensions, treated for 10 minutes with ammonium chloride solution (0.14 M, pH 7.5), were added to the monolayers in a quantity of 2×10^7 cells in 2 ml medium. The separation procedure was performed according to the method of Wekerle et al. (1972). Adsorption time was 3 hours at 37°C. Controls were run at 4°C.

Antibody-mediated cytolysis. Antibody-mediated lysis of virus-infected cells was performed as described by Kibler and ter Meulen (1975).

Cold-cell inhibition assay. The assay was performed according to the method of Herberman et al. (1976). Inhibition was calculated as follows:

$$\% \text{ Inhibition of specific lysis} = \frac{\% \text{ specific lysis in immune control} - \% \text{ specific lysis in presence of inhibitory cells}}{\% \text{ specific lysis in immune control}} \times 100.$$

Cell-mediated cytolysis. Various numbers of lymphocytes from sensitized mice and control mice were incubated with a constant number (1×10^4) of ^{51}Cr-labeled target cells (Wagner 1973). Specific ^{51}Cr-release was determined (Koszinowski and Thomssen 1975) using the formula:

$$\% \text{ Specific lysis} = \frac{^{51}\text{Cr-release by immune cells} - ^{51}\text{Cr-release by normal control}}{\text{maximal } ^{51}\text{Cr-release}} \times 100.$$

The standard deviation (S.D.) was calculated from at least a triplicate assay. Some data are given without S.D. because, under the test conditions used, the S.D. values of percent lysis were less than 5%.

RESULTS

Cold-cell Inhibition and Monolayer Adsorption

Inhibition tests with cold target cells were performed to test which antigens have to be expressed for specific lysis. To the test assay consisting of 1×10^4 ^{51}Cr-labeled target cells and 1×10^6 effector cells, different amounts of nonlabeled target cells infected with different viruses were added. In the concentration of 50 inhibitory cells to one target cell, inhibition of 91.0% could be achieved when syngenic cells infected with the sensitizing virus were added (Table 1). Normal syngenic cells or syngenic cells infected with unrelated viruses showed no inhibitory activity. Allogenic cells infected with the sensitizing virus had only slight inhibitory activity.

Since in cold third-party cell experiments blocking of specific lysis is measured, a hypothetical recognition process without subsequent lysis cannot be tested. Requirements for specific recognition can be tested by adsorption of sensitized effector cells on monolayers; this has been shown after sensitization to alloantigens (Golstein et al. 1972).

Table 1. Inhibition of Specific Lysis by Cold Inhibitory Cells

Third-party cells infected with virus	Percent inhibition of lysis[a] of vaccinia-virus-WR-infected L929 cells
L929	7.1 ± 3.2
L929 vaccinia virus WR	91.0 ± 1.4
L929 vesicular stomatitis virus	3.8 ± 2.8
L929 LCM virus	14.8 ± 1.6
P-815-X2 vaccinia virus WR	18.7 ± 3.1

[a] Effector cells derived from a pool of six C3H mice sensitized to vaccinia virus strain WR 6 days previously. Mean of percentage ^{51}Cr-release ± S.D. of four tubes per group. Specific lysis of target cells without cold cells added, 52.0%. Ratio of attacker to target cells, 100:1; ratio of cold inhibitory cells to target cells, 50:1.

Results show that adsorption of virus-sensitized T cells is possible on syngenic monolayers infected with the sensitizing virus (Table 2). If virus-specific effector cells have two receptors, one for syngenic H-2 and one for the viral antigen, adsorption should be possible on normal syngenic monolayers as well as on allogenic vaccinia-virus-infected cells. However, specific adsorption on normal syngenic monolayers or allogenic vaccinia-virus-infected monolayers was not seen, which favors the modified-self hypothesis.

Enzymatic Treatment of Target Cells and Anti-viral CMC

Target cells were treated with enzymes to investigate the necessity of H-2 antigenic determinants for anti-viral CMC. The effect of papain and VCN on the amount of surface antigens directly after treatment and 4 and 24 hours later was controlled by indirect immunofluorescence. H-2, as well as virus-specific fluorescence, was reduced or even abolished directly after treatment. Repetition of immuno-

Table 2. Adsorption of Vaccinia-virus-specific Effector Cells

Macrophage monolayers used for adsorption	Percent specific lysis of vaccinia-virus-infected C3H macrophages	Percent change relative to control
Control	17.4 ± 1.3	100
C3H	16.3 ± 2.4	94
C3H vaccinia-virus-infected	8.1 ± 2.9	47
DBA/2 vaccinia-virus-infected	18.9 ± 1.6	109
C3H vaccinia-virus-infected (4°C control)	18.6 ± 2.3	107

Supernatant (nonadherent) lymphocytes were collected from macrophage monolayers after 3 hr incubation at 37°C on a rocker platform. Attacker-to-target-cell ratio, 50:1; assay time 8 hr.

Table 3. Antibody-mediated Lysis of Vaccinia-virus-infected Cells after Treatment with Enzymes

Enzyme concentration[a]	Papain	VCN
Control	13.6 ± 1.8	8.8 ± 1.3
5	24.6 ± 0.2	13.9 ± 2.4
10	33.7 ± 1.4	9.3 ± 2.3
20	24.4 ± 1.8	14.2 ± 2.4
50	23.4 ± 0.3	15.6 ± 3.6
100	29.1 ± 2.5	66.8 ± 2.1

L929 were infected 6 hr before enzymatic treatment; ^{51}Cr-release test was performed 4 hr after treatment; antiserum to vaccinia virus (No. 8) originated from rabbits, and complement originated from guinea pigs. Assay time was 4 hr. Spontaneous ^{51}Cr-release of enzymatically treated cells, 20–25%. Each value represents the mean of four tubes ± S.D.

[a] Papain, μg/ml; VCN, u/ml.

fluorescence (IF) after 4 and 24 hours indicated resynthesis of viral antigens but, due to host-cell protein synthesis inhibition, not of H-2 antigens. Enzymatic treatment of noninfected target cells was followed by resynthesis of H-2 antigenic determinants 4 to 24 hours later. VCN treatment had no effect on either H-2 or virus-specific determinants; IF was even found intensified.

Results of IF were repeated in complement-dependent antibody-mediated lysis using an anti-vaccinia serum and infected target cells 4 hours after enzymatic treatment. The results verify the IF observations (Table 3). Virus-specific antigens are resynthesized and lysis is even increased after treatment with high doses of enzymes, especially in the case of VCN.

Enzymatic activity on H-2 antigenic determinants was tested. Absorption studies were performed because concentration differences of surface antigens cannot be distinguished easily by antibody-mediated lysis (Lesley et al. 1974). Equal numbers of nontreated and enzyme-treated (4 hr previously) infected target cells were used for absorption of an H-2 alloantiserum. Absorbing capacity was demonstrated by comparing the cytolytic activity of the alloantiserum before and after absorption. The nearly identical slopes of cytolytic activity of absorbed and nonabsorbed serum after papain treatment of absorbing cells (Fig. 1) demonstrated the reduced capacity of these cells to adsorb H-2 alloantiserum. VCN-treated cells, however, had nearly the same absorbing capacity as non-enzyme-treated cells. This means that papain is effective in reducing H-2 antigenic sites. The reduction of H-2 antigenic determinants is constant when vaccinia-virus-infected target cells are treated.

T cells sensitized to vaccinia virus were incubated with enzyme-treated or nontreated infected and noninfected target cells. Enzymatic treatment influenced lysis of syngenic infected cells. Removal of sialic acid resulted in enhanced CMC. Digestion of target cells with papain diminished the lysis of target cells (Fig. 2).

To prove the efficiency of enzymatic digestion on H-2 antigenic determinants and to exclude possible toxic activities of enzymes on the effector cells, uninfected L929 cells were subjected to papain treatment in both the same and higher concentrations than those of infected target cells. These cells were incubated with allogenic anti-H-2^k killer T cells for either 5 or 10 hours. The results (Fig. 3) show that papain treatment does in fact reduce the sensitivity of target cells to lysis by T cells directed to H-2 antigens. Since noninfected L929 cells are less fragile than infected cells, higher concentrations of papain could be used. Nonspecific effects of enzyme on effector cells could be excluded by the 10-hour assay. During this incubation period, H-2 antigenic determinants are resynthesized (Schwartz and Nathenson 1971), and effector cells are effective under these conditions. This means that T-cell-mediated lysis of virus-infected cells is dependent on expression of H-2 antigenic determinants on these cells. Viral surface antigens do not suffice for virus-specific CMC.

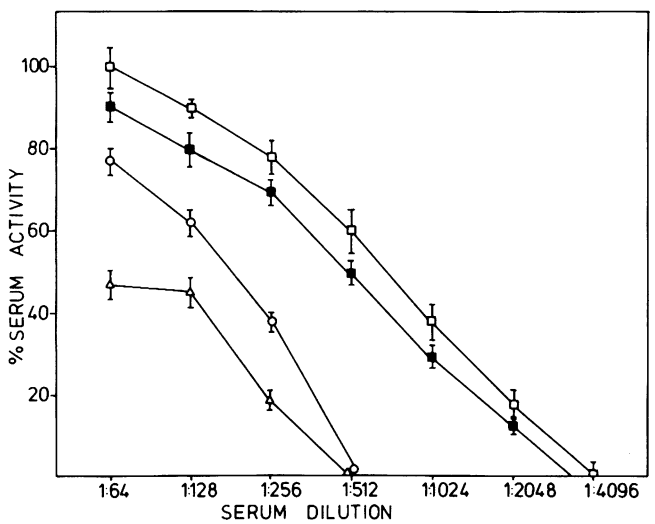

Figure 1. Residual cytotoxicity of anti-H-2^k alloantiserum after absorption on enzymatically treated cells. (□——□) Cytotoxic activity of the nonabsorbed serum control; (△——△) residual cytotoxic activity after absorption of 0.5 ml serum on 5×10^7 vaccinia-virus-infected L929 cells; (○——○) absorption on 200 U/ml VCN-treated infected L929 cells; (■——■) absorption on 200 μg/ml papain-treated infected L929 cells. The plateau activity of the anti-H-2^k serum (44.8 ± 3.2) was taken as 100%. Data represent the mean of at least four tubes ± S.D.

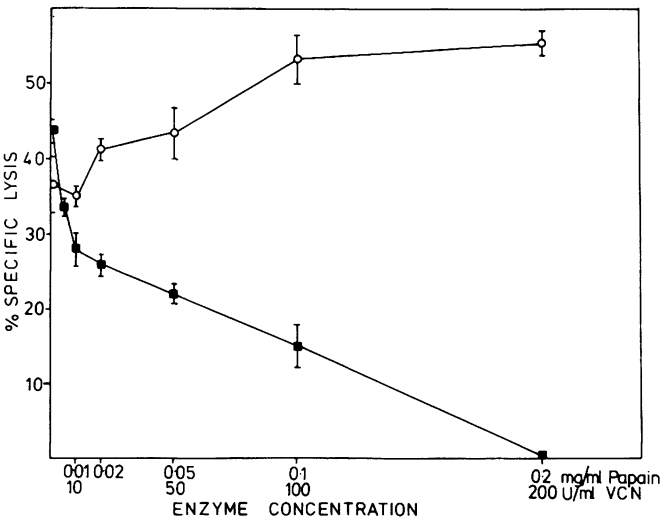

Figure 2. Alteration of cell-mediated lysis after enzymatic treatment of target cells. The figure represents data from four tubes ± S.D. (○——○) VCN-treated target cells (background lysis of enzyme-treated cells, 18–23%; specific lysis of nontreated infected L929 cell controls, 37.2%); (■——■) papain-treated target cells (background lysis of treated cells, 29.3%; specific lysis of nontreated infected L929 cell controls, 44.0%). Ratio of attacker cells to target cells was 100:1; assay time, 16 hr.

Role of Early Viral Surface Antigens in Cellular Immune Response to Vaccinia Virus

Experiments were performed to test the minimal "alterations" that must be induced on cell surfaces to induce specific sensitization or to render cells sensitive to virus-specific T-cell-mediated lysis. The virus strains we put into test varied in the expression of viral surface antigens. Characteristics of the three strains used are given in Table 4. Strain WR was positive for early and late surface antigens, strain DIs did not propagate in mouse cells but it did induce early surface antigens, and strain Elstree gave mixed hemagglutination of the single-cell type (Ito and Barron 1972) with an anti-early-surface-antigen serum.

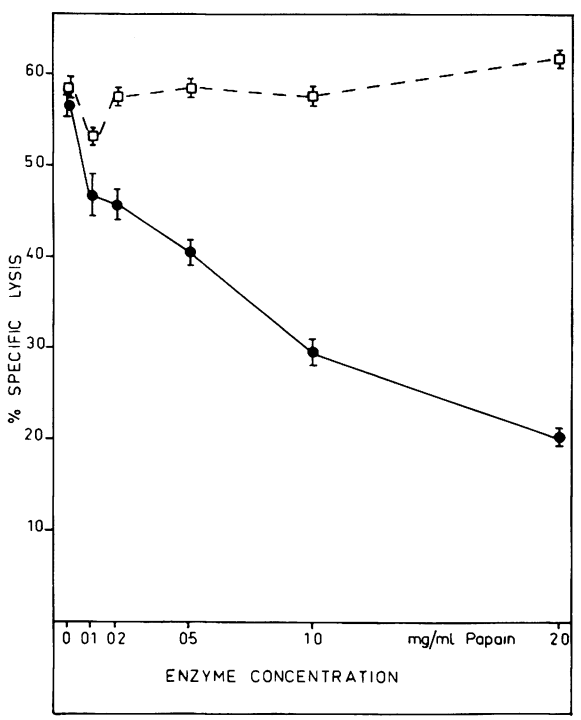

Figure 3. Influence of target-cell treatment with papain on allogenic CMC: (●——●) 5-hr assay (background lysis, 12.5–18.3%; percent specific lysis of nontreated control target L929 cells, 56.4%); (□——□) 10-hr assay (background lysis, 17.5–23.9%; percent specific lysis of nontreated control target L929 cells, 63.6%). Attacker cells were DBA/2 lymphocytes sensitized in vitro to C3H lymphocytes for 5 days. Ratio of attacker cells to target cells was 20:1.

Spleen lymphocytes of C3H mice sensitized to any of the three virus strains killed all infected target cells (Table 5). Also, lymphocytes from mice sensitized with a low dose of DIs were active, despite the fact that virus propagation did not take place in vivo. This means that DNA replication and production of late surface antigens is not a prerequisite for cellular anti-vaccinia-virus sensitization. Lymphocytes from mice sensitized to DIs or Elstree showed cross-killing of their relevant infected target cells. Although early antigen expression is sufficient, our data give no clear-cut results about the role of late surface antigens, since strain Elstree is only partially defective in production of early surface antigens.

Inhibition of Antiviral CMC by Specific Antibodies

Cytolytic activities against DIs-infected cells suggest CTL activities against virus-induced early surface antigens. Different antiviral sera were prepared to control these results in inhibition assays. These antibodies were tested for inhibitory activities in the antiviral CMC using L929 cells infected with vaccinia virus strain WR as targets and spleen cells from strain-WR-sensitized C3H mice. Table 6 gives the characterization of the antisera and their activity on antiviral CMC. Significant inhibition of CMC was seen with a serum obtained from rabbits after virus infection (No. 8) and anti-ES serum. Anti-LS serum and

Table 4. Characteristics of Vaccinia Virus Strains

Strain	DNA replication in L929 cells[a]	Cytopathic effects in L929 cells	Titer of virus in test[b] ($TCID_{50}$)	Indirect hemagglutination with anti-ES	Indirect IF on surfaces	
					anti-ES	anti-LS
WR	yes	yes	10^6	positive	+++	+++
Elstree	yes	yes	10^6	single-cell type	+	++
DIs	no	no	10^5	positive	++	−

[a] Tested with DAPI and by indirect IF with anti-VA.
[b] Strains WR and Elstree were propagated and titrated in VERO cells; strain DIs was propagated in embryonated eggs (chlorioallantois membrane) and titrated on primary fibroblasts of 6-day-old chicken embryos.

serum directed against antigens of the virus particle (anti-VA) had no significant inhibitory activities. The inhibition test with 1 ODU of inactivated vaccinia antigen, after addition of about 1.2×10^{10} inactivated vaccinia elementary bodies, was negative. Therefore, the virus-specific antigen receptor site on the cytolytic T cell does not seem to recognize antigens of the virus particle.

Table 5. Antiviral CMC of CTL from Mice Sensitized with Different Vaccinia Strains

Sensitization	Percent specific release[a] from L929 cells infected with		
	DIs	WR	Elstree
1×10^6 $TCID_{50}$ WR	45.0	41.8	15.7
1×10^6 $TCID_{50}$ Elstree	18.8	27.3	15.1
1×10^5 $TCID_{50}$ DIs	27.6	14.2	N.D.

Lymphocytes were derived from C3H mice sensitized 6 days previously.
[a] Background lysis ranged between 20–25%; assay time was 14 hr; ratio of attacker cells to target cells was 100:1; N.D. = not done.

Table 6. Activities of Anti-vaccinia Serum

Serum	Indirect cell-surface IF[a]		CA[b]	NT[c]	Percent specific lysis[d]
	WR	DIs			
Anti-vaccinia (No. 8)	+	+	1:1024	1:32	15.0[e]
Anti-ES	+	+	1:1024	1:<4	17.0[e]
Anti-LS	+	−	1:512	N.D.	23.7
Anti-VA	−	−	1:<4	1:256	25.8
Normal serum					24.8
None					27.4
1.2×10^{10} vaccinia particles					32.4

[a] Indirect IF was tested on L929 cells infected with vaccinia strains WR and DI's 8 hr previously.
[b] CA = cytolytic antibody assay. Cytolytic activity was tested on L929 cells infected with vaccinia strain WR in a 4-hr ^{51}Cr-release assay.
[c] N.D. = Neutralizing test. Neutralizing activity was tested on VERO cell monolayers.
[d] Lymphocytes were derived from C3H mice sensitized 6 days previously with vaccinia virus strain WR. Ratio of attacker cells to target cells, 50:1; incubation time, 12 hr; background lysis, 22.8%.
[e] Significantly reduced specific ^{51}Cr-release ($P < 0.05$).

DISCUSSION

Cold-cell inhibition experiments were performed to look for blocking activities of cells bearing different virus-induced "modifications." Only syngenic vaccinia-virus-infected cells had blocking activities. No effect was produced by adding allogenic vaccinia-virus-infected cells or syngenic cells infected with other viruses. The necessity of H-2 homology for effective blocking of CMC of "modified" cells has also been shown by others (Shearer et al. 1975; Rehn et al. 1976). The aim of the absorption studies was to discriminate between cytolysis and recognition. Monolayer absorption can effectively fractionate antigen-sensitive cells (Wekerle et al. 1972; Golstein et al. 1972). If virus-specific CTL have two antigen receptors, one for syngenic H-2 and one for the viral antigen, specific adsorption should be possible on normal syngenic monolayers or on allogenic vaccinia-virus-infected cells. Lysis would occur only if both antigens are expressed on the same cell. The finding that specific adsorption takes place only on vaccinia-virus-infected monolayers sharing K and D structures with the effector cells is very much in accord with the altered-self hypothesis (Doherty et al. 1976), which requires only one antigen receptor site on T cells for recognition and cytolysis. There is no independent recognition of both single antigenic determinants, or at least a very low affinity of the receptors.

It was next of interest to investigate the reactivity against target cells bearing viral antigens but reduced or even abolished H-2 antigenic sites. These target cells could be prepared since vaccinia virus infection inhibits the host-cell protein synthesis (Moss 1968). H-2 antigenic sites were not reexpressed after enzymatic treatment while synthesis of viral surface antigens took place. The main results from these experiments were that viral antigenic determinants which are detectable by humoral antibodies (immunofluorescence, antibody-mediated antiviral cytotoxicity) did not suffice for the effector function of antiviral CTL.

CMC of virus-infected target cells was found reduced after papain treatment but not after VCN treatment. As has been shown by Sandford and Codington (1971), removal of sialic acid has no

inhibitory activity on cellular recognition of H-2 antigenic determinants while H-2 can be dissolved from cell membranes by papain treatment (Nathenson and Davies 1966; Yamane and Nathenson 1970). It could be demonstrated that the inhibition of CMC acts on the target-cell level. After treatment of normal L929 cells with papain, cytolysis by allogenic killer cells sensitized against H-2^k antigens was found reduced, but under test conditions allowing resynthesis of H-2 antigens (Schwartz and Nathenson 1971), there was no inhibition of effector-cell activity. This rules out unspecific inhibitory effects of any active enzyme possibly remaining on the effector cells.

These results do not support the finding (Dennert 1975) that the T cell recognizes the modifying hapten on the cell surface. Cytolytic T cells specific for new antigens on cells also need H-2 antigenic sites on the target cell for their effector function. Reduction of H-2 by enzymatic treatment or by capping with alloantiserum (Koszinowski and Ertl 1975; Schmitt-Verhulst et al. 1976) decreases the ability of virus-infected target cells to be lysed by antiviral CTL. This corresponds to observations (Lightbody and Bach 1973; Brondz et al. 1973) that proteolytic treatment reduced allogenic CMC. Thus the virus-specific CTL act in an analogous way to the alloantigen-specific CTL, which are dependent on the concentration of K- or D-region products on the target cell (Alter et al. 1973). One can assume that CTL can only be sensitized to and recognize new antigenic determinants which interact with antigens coded by genes of the K or D region of the major histocompatibility complex (MHC).

Investigating the minimal requirements of cell-surface alterations by a virus infection, it was found that early surface antigens, induced by vaccinia virus, give rise to the generation of anti-vaccinia CTL. This could be demonstrated using a conditional lethal mutant strain of vaccinia virus (Tagaya et al. 1961) which does not replicate in mice. The essential role of early viral antigens for induction of cellular and humoral immune mechanisms has been suggested previously (Ueda and Tagaya 1973). Also, in the effector phase, target-cell infection with strain DIs was sufficient for induction of cell-mediated specific lysis.

The inhibition experiments with different antiviral sera outline the significance of early antigens. Using sera against different viral antigens, the neutralizing activity could be separated from CTL inhibitory properties. Contact with complete virus particles is not necessary; they do not inhibit the cytotoxic interaction, nor does the injection of inactivated virus particles give rise to CTL production (unpubl.). CTL recognition is therefore specific for plasma membrane alterations but not for antigens of the virus surface.

If CTL have biological functions in vivo, sensitization and reactivity against virus-induced early antigens seem to be advantageous. They are expressed as early as 1 hour after infection (Ueda et al. 1969), whereas maturation of complete infective virus needs several hours. In the reaction against alterations of the cell membrane induced by early antigens, the CTL could prevent synthesis of virus particles and, in later stages of infection, the spreading of virus.

Vaccinia-virus-specific early surface antigens, whose role in virus replication is not clear, perform the functional H-2 antigenic alteration which gives rise to vaccinia-virus-specific CMC. Characterization of the relevant polypeptides (Esteban and Metz 1973; Polisky and Kates 1975) that are synthesized very early after infection will help to clarify the biochemical basis of H-2 antigen "alteration."

SUMMARY

Specific recognition of antigens by cytolytic T lymphocytes sensitized to vaccinia virus was tested by monolayer adsorption. Adsorption was possible only on monolayers also expressing syngenic H-2 as viral antigens present during the sensitization phase.

Vaccinia-virus-infected target cells were subjected to papain and neuraminidase treatment. H-2 antigenic determinants could be removed by papain treatment. Due to virus-specific inhibition of host-cell protein synthesis, reexpression of H-2 antigenic determinants did not take place, but viral surface antigens were resynthesized. Susceptibility of target cells to T-cell-mediated lysis was decreased after papain treatment.

Substrains of vaccinia virus were used in order to define the minimal changes induced by vaccinia virus necessary for T-cell sensitization in vivo and target-cell lysis in vitro. When the immune response to a conditional lethal mutant strain of vaccinia virus was investigated, it could be demonstrated that expression of early surface antigens is sufficient for induction of the cellular immune reactions. These data were confirmed by inhibition studies with virus-specific antisera.

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